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CHEMO-IMMUNOLOGICAL STUDIES ON LOCALIZED INFECTIONS.[1]

First Paper: Action on the Pneumococcus and Its Experimental Infections of Combined Sodium Oleate and Antipneumococcus Serum.

By RICHARD V. LAMAR, M.D.

With an Introduction by

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INTRODUCTION.

The phenomenon of recovery from any local bacterial infection has not been fully explained, and it is not wholly accounted for by the several activities of blood serum and phagocytes, which have often been viewed as gradually overcoming and removing the offending bacterial agents. Indeed, this restricted view has never been fairly established either by direct observation upon affected human beings or animals, or through experiment; and it leaves out of account the effects of certain definite chemical substances, other than antibodies, which are always present in a focus in which tissues and cells are disintegrating. That some of these chemical substances are injurious to bacteria and even to somatic cells we now know, and it has been found possible to define more precisely the chemical nature of part, at least, of the injurious chemicals.

It has long been known that certain somatic cells yield upon extraction bactericidal substances that are neither complements nor amboceptors. Thus the long series of investigations, beginning with those of Buchner[2] and Denys,[3] including those of Hahn,[4] Schat-

[1] Received for publication, August 1, 1910.

[2] *Arch. f. Hyg.*, 1890, x, 84.

[3] *La Cellule*, 1893, ix, 335; 1894, x, 5.

[4] *Arch. f. Hyg.*, 1895, xxv, 105.

tenfroh,[5] van der Velde,[6] Bail,[7] Moxter,[8] and Löwit,[9] and terminating with those of Pettersson[10] and Zinsser,[11] have eventuated in proving that the substances extracted or expressed from leucocytes are capable of destroying bacteria of a wide series ranging from the pyogenic cocci, on the one hand, to the bacilli typhosus, coli, and anthracis, on the other. The distinguishing characteristics of these intracellular bactericidal substances are their thermostability and capacity to act independently of the presence of alkaline salts in the medium.

These extractive substances, obtained either directly from cells or after autolysis, have been shown by Conradi,[12] Korschun and Morgenroth,[13] Levaditi,[14] and Noguchi,[15] to be not only bactericidal, but often hemolytic as well, and to differ still further from the thermolabile antibodies in being alcohol-soluble. Very recently Noguchi[16] has determined that the activity of these extracts depends largely, if not wholly, upon their content in certain higher unsaturated fatty acids or their alkaline soaps. That soaps occur in inflammatory foci has been shown by Klotz;[17] and the origin of the fatty acids and soaps is readily accounted for since Achalme[18] has proved the presence of lipase in such foci. That neutral fat and even the higher phosphorized fats or lecithin complexes occur in disintegrating tissue and exudate is well known; and under the influence of the lipase, which Bergel[19] believes to be especially abundant in the mononuclear leucocytes or lymphocytes,

[5] *Arch. f. Hyg.*, 1897, xxxi, 1; 1899, xxxv, 135.
[6] *Centralbl. f. Bakteriol., 1te Abt.*, 1898, xxiii, 692.
[7] *Arch. f. Hyg.*, 1897, xxx, 348.
[8] *Deutsch. med. Wchnschr.*, 1899, xxv, 687.
[9] *Beitr. z. path. Anat. u. z. allg. Path.*, 1897, xxii, 172; *Centralbl. f. Bakteriol., 1te Abt.*, 1898, xxiii, 1025.
[10] *Centralbl. f. Bakteriol., Orig.*, 1905, xxxix, 423, 613; 1908, xlvi, 405.
[11] *Jour. Med. Research*, 1910, xxii, 397.
[12] *Beitr. z. chem. Phys. u. Path.*, 1902, i, 193.
[13] *Berl. klin. Wchnschr.*, 1902, xxxix, 870.
[14] *Ann. de l'Inst. Pasteur*, 1903, xvii, 187.
[15] *Biochem. Ztschr.*, 1907, vi, 327.
[16] *Loc. cit.*
[17] *Jour. Exper. Med.*, 1905, vii, 633.
[18] *Compt. rend. Soc. de biol.*, 1899, li, 568.
[19] *München. med. Wchnschr.*, 1909, lvi, 64; 1910, lvii, 1683

these fats are decomposed and their fatty acids liberated. Certain tissues which have undergone autolysis *intra vitam* yield an alcoholic extract of high hemolytic activity, as has been shown for the liver in acute yellow atrophy, toluylendiamin and phosphorus poisoning, by Joannovics and Pick,[20] Ehrmann and Stern,[21] and Jacoby,[22] and this unusual potency is ascribed to the presence of free, higher unsaturated fatty acids which are derived from cleavage of the lecithin-like complexes. Dr. Lamar has extracted relatively large quantities of such lecithin-like bodies or lipoids and fatty acids from the human lung, the seat of resolving lobar pneumonia.

The quantity of bactericidal substances extractable from leucocytes *in vitro* must be a small measure of those which may be liberated from leucocytes in an inflammatory focus. These extractable substances represent merely the preformed bodies and are minimal in amount. While it is probable, it has yet to be proved, that they consist of soaps. The leucocytes in an inflammatory focus, on the other hand, suffer successive death and disintegration either through the action of bacterial poisons—leucocidins—or because of lack of nutriment, and then yield through autolysis and lipolysis the fatty complexes out of which fatty acids and soaps are produced. The quantity of bactericidal and hemolytic substances yielded by cells in process of autolysis is relatively considerable. The disintegration of leucocytes and tissues which results from a local bacterial infection can, therefore, be viewed as a process that is not entirely to the advantage of the parasitic agent, but is also of use to the economy in assisting to overcome the bacteria, since the cells brought to death and disintegration by the parasites yield certain definite chemical substances that themselves exert a destructive action upon the infecting bacteria.

Turning now to the subject of the pneumococcus, with which this paper deals, and considering the typical form of pneumococcus inflammation presented by lobar pneumonia, it should first be stated that the pneumococci present in the resolving exudate

[20] *Ztschr. f. exper. Path. u. Therap.*, 1909, vii, 185; *Berl. klin. Wchnschr.*, 1910, xlvii, 928.

[21] *Berl. klin. Wchnschr.*, 1910, xlvii, 282.

[22] *Berl. klin. Wchnschr.*, 1910, xlvii, 677.

undergo a progressive diminution, both in number and in virulence, as the process of resolution proceeds. This decrease in the number of pneumococci is not produced by phagocytosis, which occurs, as a rule, but slightly; and test tube experiments have shown that the pneumococcus is little, or not at all, subject to serum-lysis; hence some other mechanism must be invoked to explain their removal. Many years ago Welch[23] observed that the resolution of the pneumococcic exudate was accompanied by the lysis of the pneumococci within their capsules, and that all stages of the chromatolysis of the organisms, up to complete disappearance, could be followed under the microscope. What the nature of the agency is that brings about this form of solution of the pneumococci within their capsules has not been determined. Now it becomes significant, as Dr. Lamar's investigation shows, that pneumococci which have been exposed to the action of weak solutions of sodium oleate become so altered in texture that, while still viable in cultures, they are subject to lysis or solution by means of the body fluids and particularly by means of an antipneumococcus serum *in vitro* or *intra vitam.*

Such an assumption as that the alteration in virulence and number suffered by pneumococci during resolution of a lobar pneumonic exudate depends in part upon the action of the fatty acids and soaps of the exudate, would, in view of the facts presented, have some justification; and this assumption would be further strengthened by the additional fact ascertained by Dr. Lamar, that the viable, soap-treated pneumococci have had their virulence diminished by the treatment.

On the other hand, an obstacle immediately appears in the way of this highly satisfactory explanation. Noguchi[24] and von Liebermann[25] have separately noted that hemolysis and bacteriolysis by the unsaturated fatty acid soaps are inhibited by the protein of the serum; and the question, therefore, arises whether the acids and soaps produced in the resolving exudate could come to act injuriously upon the bacteria and in this case upon the pneumococci. No definitive reply can be made at the moment to this question,

[23] *Johns Hopkins Hosp. Bull.*, 1890, i, 73.
[24] *Loc. cit.*
[25] *Biochem. Ztschr.*, 1907, iv, 25.

since we are still too ignorant of the interaction of chemical substances within a resolving exudate. But we may still consider factors that possibly suffice to overcome this initial impediment to the action of the soaps, among which are the proximity of the bacteria to the nascent fatty acids and soaps and the natural occurrence within the exudate of chemical bodies that have the effect of removing the protein inhibition similar to that described for boric acid in the experiments of Dr. Lamar.

The complexity of the defensive processes invoked against bacteria is further emphasized in a suggestive way by the striking part taken by the antipneumococcus serum in the experiments. The action of combined sodium oleate and immune serum upon the lysis of the pneumococci and upon infection with that organism indicates a highly important property of immune serum. The destructive and protective effects described in connection with the combination are probably not simply those of summation of two independent injurious agencies, but may well be the expression of a new and specially acquired property of the antiserum, since the normal serum fails to produce perfect and complete lysis of the soaped pneumococci. Moreover, it has been shown clearly, especially by Neufeld[26] and his associates, that, at the time of the appearance of the crisis in pneumonia, antibodies are demonstrable within the blood; thus, it would appear that the conditions afforded by the inflammatory exudate for the interaction of the soaps formed within the exudate and the bacteria there present may come to be favorable to the destruction of the pneumococci.

We entertain the opinion that our knowledge of the phenomena of local destruction of infecting bacteria may be promoted by a more comprehensive study of the chemistry of the process and by declining to confine attention to the antibodies and phagocytes exclusively. The purpose of the present paper, and of those which it is expected will follow, is to attempt to define the part played in destroying bacteria by the more definite chemical bodies occurring within local inflammatory foci, especially those undergoing resolution, and to put to a practical test, in promoting recovery from experimental infections, the facts ascertained in the course of the investigation.

[26] *Arb. a. d. k. Gsndhtsamte.*, 1910, xxxiv, 166.

ACTION ON THE PNEUMOCOCCUS AND ITS EXPERIMENTAL INFECTIONS OF COMBINED SODIUM OLEATE AND ANTIPNEUMOCOCCUS SERUM.

CONDITIONS OF PNEUMOCOCCUS INFECTION.

The state of our knowledge of the conditions of pneumococcus infection will be indicated by the brief review of the literature that is to follow. The effects of the entrance of bacteria into an animal body are considered to depend chiefly upon the susceptibility of the animal and the virulence of the invading bacteria. These expressions are obviously merely relative and bear a reciprocal relationship, yet at the extremes the states of susceptibility and virulence are well defined and easily appreciated. The conditions are well illustrated by the manner of reaction of different animals to the pneumococcus. Certain animals, under normal conditions, represent one extreme of entire insusceptibility to infection with the pneumococcus, and others present the opposite extreme of high susceptibility to its effects. Thus, the pigeon is entirely resistant to infection, while the goat, dog, sheep, guinea pig, rat, mouse, and rabbit, as well as man, must be ranked among the susceptible animals. The reactions of the susceptible animals vary. There may be only a slight local inflammation, or a severe local inflammation with or without septicemia, or an immediate fatal septicemia following a slight local reaction. Experimental evidence exists to the effect that the susceptibility of the dog to experimental infection with the pneumococcus is similar to that of man to spontaneous infection. That is, in man and the dog, pneumococcus infections are usually of the nature of severe local reactions, which only exceptionally are attended by true septicemia, as distinguished from the early transitory bacteremia. But in man, as in susceptible lower animals, the effects depend, in considerable part, upon the degree of virulence of the pneumococci. The pneumococci which thrive saprophytically in the buccal cavity of human beings are apparently, as a rule, harmless, and when cultivated from this source the organism has little pathogenicity, sometimes none, for the highly susceptible mouse and rabbit. However, when the pneumococcus is associated with local inflammations in human beings, of which

many varieties occur, both as regards situation and severity, the pneumococci cultivated from the lesions exhibit all degrees of pathogenicity for these very susceptible animals. The organisms obtained from most of the local inflammations are not highly pathogenic, but those obtained from pneumonic lesions are sometimes, although not invariably, of high potency.

The basis of these differences in susceptibility of animals and in virulence of different strains of pneumococci has been sought from the time of the earliest work by the pupils of Metchnikoff to that of the recent studies of Hektoen[27] and his pupils, especially in relation to phagocytosis. Tchistovitch[28] concluded from his experiments that the state of refractoriness or susceptibility to infection depended wholly upon whether the leucocytes of the animals did or did not ingest and destroy the pneumococci. Mennes[29] further noted that an animal artificially immunized against the pneumococcus resisted infection because its leucocytes exercised increased power to take up the organisms. Moreover, his studies of the virulence of different strains of the diplococcus led him to conclusions similar to those arrived at by Marchand in his studies of the streptococcus, namely, that the avirulent pneumococci are readily taken up by phagocytes, while the virulent are not. He therefore concluded that a pneumococcus produces infection because it possesses the power to resist destruction by leucocytes, a view which has been confirmed and accepted by many later workers. Recently Strouse[30] has added that virulence depends in part upon the ability of the pneumococcus to grow in the body of the animal, and not wholly upon its resistance to phagocytosis.

Much attention has been given to the study of the causes that underlie the resistance to phagocytosis shown by virulent pneumococci. Marchand[31] ascertained that the killing of the organisms by heat and the treatment of them with various chemical substances did not render the previously virulent organisms subject to phagocytosis, from which he concluded that the determining factor was

[27] *Jour. Infect. Dis.*, 1906, iii, 446, 731; 1907, iv, 285; 1908, v, 273.
[28] *Ann. de l'Inst. Pasteur*, 1890, v, 285.
[29] *Ztschr. f. Hyg.*, 1897, xxv, 413.
[30] *Jour. Exper. Med.*, 1909, xi, 743.
[31] *Arch. de méd. expér. et d'anat. path.*, 1898, x, 253.

some physical state, and not the chemical composition, of the pneumococci. Recently, however, Rosenow[32] has attributed the resistance to phagocytosis offered by virulent pneumococci to a substance called by him "virulin," which he believes he has shown to be present in the virulent organisms not subject to phagocytosis and to be absent in the avirulent ones; and, moreover, that the substance may be separated from the organisms containing it through autolysis. Similarly, Tchistovitch and Jourevitch[33] have stated that virulent pneumococci may be rendered subject to phagocytosis by merely washing them with salt solution which removes a substance called by them "antiphagine," upon which they believe the resistance to phagocytosis depends.

Studies of the action of immune or antipneumococcus serum have succeeded in establishing its biological properties and in determining that its protective value against infection is slight. Such immune sera exert only a slight agglutinating action upon the pneumococci and are devoid, or nearly so, of antitoxic and bactericidal action. They differ from the corresponding normal sera chiefly in their content in bacteriotropin or opsonin, through which they promote phagocytosis to a greater degree than normal serum does. It is true that when immune sera are injected into the body of certain susceptible animals in advance of the inoculation of the pneumococci, infection is sometimes prevented, which, according to Neufeld[34] and his pupils, is the result solely of the action of the specific bacteriotropin. Yet these immune sera have proven powerless to cure an established pneumococcus infection. To this statement there is, possibly, one exception. Römer[35] believes that the antipneumococcus serum is effective in the treatment of corneal ulcers caused by the pneumococcus.

EXPERIMENTAL OBSERVATIONS.

With our present knowledge, we must view the power of an antipneumococcus serum to promote phagocytosis as the index of

[32] *Jour. Infect. Dis.*, 1907, iv, 285.

[33] *Compt. rend. Soc. de biol.*, 1908, lxiv, 1095.

[34] *Ztschr. f. Immunitätsforsch.*, 1909, iii, 159.

[35] Serumtherapie der Pneumokokkeninfektion der menschlichen Cornea, Wiesbaden, 1909.

its therapeutic activity, and we must also view the property of spontaneous phagocytosis of the pneumococci, which certain animals exhibit, as the determining cause of their refractoriness to infection. The only known exception to this is the pigeon, whose refractoriness Strouse[36] believes to be due to its normally high temperature. These considerations do not exclude the probability that there may be still other and undetermined factors concerned in the prevention of infection by the pneumococcus.

Sodium Oleate and Lysis of the Pneumococcus.—Although antipneumococcus serum possesses in itself neither bactericidal nor bacteriolytic power, we have found that when it acts in conjunction with certain chemical substances, it comes to exert both of these actions. The chemical substances which we have studied especially are the alkaline soaps of oleic acid. That soaps are bactericidal for certain bacteria, namely, the bacilli of typhoid fever, anthrax, and dysentery, has previously been shown by Noguchi.[37] We have noted that the oleates of sodium, ammonium, and neurin are bactericidal for the pneumococcus also, and that while the effects are, in general, qualitatively alike, the sodium soap is perhaps the most, and the neurin soap the least, active of the series. In the experiments to be described, sodium oleate was employed exclusively.

In the experiments virulent pneumococci have been subjected to several different concentrations of sodium oleate, alone and in conjunction with normal and immune sera. In the first place, in order to determine the effects upon the pneumococci of the soap solutions alone, they have been added to broth cultures, and microscopical studies have been made for morphological and tinctorial changes; and, in addition, tests were made to ascertain the viability on artificial culture medium of the treated diplococci, as well as their degree of virulence for rats and mice.

The virulence of the cultures employed was maintained at a high point by daily passage through mice. The greater part of our studies was made with two cultures, designated "74" and "N.1," which throughout the period of the experiments were fatal in doses of 0.000001 cubic centimeter for a full grown mouse, and

[36] *Loc. cit.*

[37] *Biochem. Ztschr.*, 1907, vi, 327.

0.00001 cubic centimeter for a grown rat. In each experiment a culture in broth eighteen to twenty-four hours old, made directly from the heart's blood of a "passage" mouse was employed, and in each, control animals were employed to guard against any sudden change in the pathogenicity.

When sodium oleate is added to a broth culture of the pneumococcus in strengths of 1 to 0.5 per cent. of the volume, the diplococci are killed within fifteen to thirty minutes. In hanging-drop and film preparations the diplococci are seen to disintegrate slowly without swelling. To the naked eye the mixture clears only slightly and becomes viscid. This conversion of the disintegrated cocci and soap into mucin-like material is more strikingly apparent when the number of cocci in the mixture is increased, as by centrifugalizing the culture and resuspending the sedimented diplococci in broth or salt solution to one-fourth of the original volume of culture. Then nearly the whole of the mixture becomes converted into a thin translucent jelly which persists unchanged for two days. In this respect the action of sodium oleate differs strikingly from that of bile which causes complete solution of the diplococci and renders the suspension perfectly clear, transparent, and limpid.

Greater dilutions of the sodium oleate produce less profound changes in the pneumococci, and when the dilution of the soap is greater than 1 to 1,000, the pneumococci are not killed after one hour's contact. When such mixtures of soap and culture are centrifugalized at a high rate of speed the pneumococci collect in the bottom of the tube in the form of a whitish coherent slimy mass, which upon being resuspended in salt solution may be readily broken up into a fine suspension resembling the original culture.

When pneumococci which have been treated with sodium oleate in the way described and suspended in salt solution are allowed to undergo autolysis, it is found that they disintegrate more quickly than do normal pneumococci of the same origin suspended also in salt solution. While the former undergo complete disintegration in a few hours so as to leave the fluid clear, the latter are incompletely disintegrated after a lapse of two days.

If the strength of the sodium oleate is still further reduced to

dilutions of 1 to 10,000, 15,000, and 20,000, the exposed cocci are not altered in morphological appearance, staining properties, or ability to grow upon artificial culture medium, yet they still undergo autolysis with much greater rapidity than the untreated diplococci.

Experiments were made to determine whether the diplococci, which were obviously altered without being killed, so that they underwent more rapid autolysis, would also show greater capacity for phagocytosis. The experiments were made as follows:

A 0.2 per cent. solution of sodium oleate was added to broth cultures to produce a concentration of 1 to 10,000 to 1 to 20,000 of the soap. After one hour's contact at room temperature the soap and broth were removed by washing the pneumococci twice in 0.85 per cent. salt solution in a centrifugal machine of high speed. The treated pneumococci were collected and made into a fixed suspension in salt solution, which suspension was used for the phagocytic tests. These tests were made with leucocytes alone and with leucocytes plus normal serum, and immune serum. Control observations were carried out with pneumococci of the same source which had been merely washed in salt solution. No phagocytosis had occurred in any of the preparations after the expiration of thirty minutes. On the other hand, while the number of diplococci remained unchanged in the control tubes and in the mixture of leucocytes and soaped diplococci alone, the tubes containing the soaped diplococci and the normal and immune sera showed a great reduction in the number of organisms; indeed, almost all had disappeared. Hence, it appears that the combined action of soap and serum led to a marked solvent or bacteriolytic effect upon the pneumococci, and at this early period in the observations no difference was noticeable between the action of the normal and the immune serum. But at the expiration of about eighteen hours a marked difference was apparent, for the tube containing the normal serum was now turbid, as a result of the multiplication of the small number of diplococci which had escaped destruction, while the tube containing the immune serum was sterile, since all of the diplococci had been dissolved. It could now be shown that in the solution of the soaped diplococci by means of serum, the leucocytes which had been added

for the purpose of ascertaining whether the treated cocci were phagocytable played no part, since the partial solution of the soaped organisms by the normal serum, and the complete solution by the immune serum, occurred likewise in the absence of the leucocytes.

THE EFFECTS OF SOAPED PNEUMOCOCCI UPON ANIMALS.

The preceding experiments were next modified to the extent that the various mixtures, instead of being allowed to remain in test tubes, were injected into the peritoneal cavity of rats and mice. In order to follow the series of changes taking place in the peritoneal cavity and to compare them with the changes occurring in test tubes, fluid was withdrawn by means of capillary tubes at various intervals after the injection.

In the case of the soaped pneumococci there appear within the first few hours an increasing number of leucocytes, and there takes place a rapid reduction in the number of organisms injected. In the case of the control series, in which pneumococci merely washed with salt solution have been injected, there is an absence of marked leucocytic exudation and an early and rapid multiplication of the diplococci. Phagocytosis is only occasionally met with in the exudate in either series of experiments. At the expiration of about eighteen hours all the control animals are found to have succumbed to a pneumococcus septicemia, while those which have been injected with the soaped diplococci alone and with the soaped organisms combined with normal serum are very ill or dying, and the peritoneal exudate obtained by means of capillary tubes contains many pus cells and innumerable diplococci. On the other hand, the mice that received the mixture of soaped pneumococci and immune serum are not noticeably ill. The peritoneal cavity, moreover, is found to contain a very small quantity of fluid in which there are no diplococci, very few pus cells, and relatively many mononuclear, probably desquamated endothelial cells, which often contain ingested polymorphonuclear leucocytes. All the mice of the experiment die within the day except those which received the immune serum; they recover. The capacity of the immune serum to render innocuous the soaped diplococci is very great, since, when for the rat the culture is of such virulence that 0.00001 cubic centi-

meter will kill a grown rat within two days, these animals withstand under the conditions mentioned the quantity of pneumococci contained in ten to fifteen cubic centimeters of the original culture. These results will be illustrated by a protocol which follows:

March 29, 1909. The plain broth culture, which was twenty-two hours old, was made from the heart's blood of a "passage" mouse. The soap solution was freshly prepared with Merck's sodium oleate. The serum consisted of a specimen of normal goat serum and of immune goat serum, the latter prepared by injecting highly virulent pneumococci intravenously into the goat. The soap solution was added to the broth culture in such quantity as to bring about a dilution of 1 to 20,000, and the mixture was allowed to rest for one hour at room temperature before being centrifugalized. The diplococci were rapidly washed twice with 0.85 per cent. salt solution in a centrifugal machine of high speed. The sedimented organisms were finally suspended in salt solution in such a manner as to make each 0.5 c.c. of the suspension represent 15 c.c. of the original culture. The control observations were made upon pneumococci treated in all respects in a similar manner, except that they had not been subjected to the action of the sodium oleate. Microscopical examination showed no appreciable difference in the soaped and the control diplococci. Equally profuse growths were obtained from each upon glucose serum agar. The quantity of the suspensions injected into the peritoneal cavity of each rat was 0.5 c.c.

Table I summarizes the results of the experiment.

TABLE I.

		Peritoneal fluid withdrawn.			Result.
		4 hours.	8 hours.	21 hours.	
Rat 41.	Control: washed diplococci alone.	No reduction in number of cocci; few polymorphonuclear leucocytes.	Slight increase in number of cocci; more p.n.l.		Died, 17 hrs.
Rat 37.	Washed diplococci + immune serum, 0.3 c.c.	No reduction in number of cocci; few p.n.l.	Marked increase in number of cocci; many p.n.l.		Died, 19 hrs.
Rat 36.	Soaped diplococci alone.	Few cocci; very few p.n.l.	Few cocci; very few p.n.l.	Abundant growth of cocci; many p.n.l.	Died, 29 hrs.
Rat 44.	Soaped diplococci + normal serum, 0.3 c.c.	Marked reduction in number of cocci; few p.n.l.	Marked reduction in number of cocci; more p.n.l.	Abundant growth of cocci; many p.n.l.	Died, 30 hrs.
Rat 47.	Soaped diplococci + immune serum, 0.3 c.c.	Only an occasional pair of cocci; few p.n.l.	No cocci; more p.n.l.	No cocci; fewer p.n.l.	Recovered.

The animals that died presented at autopsy the usual findings from pneumococcic septicemia. Only an occasional example of phagocytosis in the peritoneal exudates.

This experiment, which is taken as an example from many of the same kind, shows clearly that the observations made *in vitro* on the action of soaps upon the pneumococci are applicable to the effects produced *intra vitam*. The conclusion, therefore, is justified that under the influence of minimal quantities of alkaline oleic acid soaps the pneumococci are so modified that they become more subject, not to phagocytosis, but to increased serum lysis, and that the effects of serum in this respect are much greater in the case of immune than in the case of normal serum. The effect, moreover, of the immune serum is to bring about the disappearance of the diplococci not by increasing phagocytosis, but by increasing lysis. Hence this experiment develops an important fact that was not ascertained in working with immune serum alone, for while the greater action of the immune serum over normal serum has, up to the present, been attributed to its greater bacteriotropic powers, it now appears that it possesses a greater lytic power as well.

THERAPEUTIC OBSERVATIONS.

Now that it has been shown that the combined effects of sodium oleate and an immune antipneumococcus serum suffice to protect rats which otherwise succumb to pneumococcus infection, when the immune serum is injected simultaneously with the soaped diplococci, the question arises whether any protection can be secured by injecting the immune serum at a later period and, hence, after infection has already been accomplished. When the above experiment is repeated with the difference that the injection of immune serum is made after that of the soaped diplococci, it has been found that it is possible to prevent infection when the immune serum is injected within about one hour after the pneumococci, but not when it is injected later and after multiplication has already begun. Since the pneumococci employed have such a high degree of virulence, and the progeny of the soaped opsonins are not subject to serum lysis, this result was predictable.

It next became necessary to ascertain whether a favorable result

could be achieved when mixtures of soap and immune serum were injected into animals already infected with untreated virulent pneumococci. In planning experiments of this kind it was necessary first to take into account the already ascertained fact that bacteriolysis and hemolysis by means of soaps are inhibited in the presence of an excess of protein in the form of serum. Thus, if the culture of pneumococci, serum, and solution of soap be mixed together at once, the diplococci are not acted upon by the soap and hence are not prepared for solution, but multiply either in the test tube or in the body of the injected animal, in the latter instance causing death. If, therefore, any success in this line of experiment is to be achieved, a method must be found for keeping apart the soap and the serum at least until the soap has had time to unite with and act upon the pneumococci.

THE PREVENTION OF PROTEIN INHIBITION OF SODIUM OLEATE.

In considering the factors that act immediately in inhibiting the action of the soap upon the diplococci in the presence of serum, the first possibility thought of related to the calcium salts, which, by reacting with the sodium oleate might give rise to calcium oleate. As calcium oleate is insoluble, it would fail to act upon and alter the pneumococci. An effort was made, therefore, to remove the calcium of the serum by means of sodium oxalate; this oxalated serum was mixed with the culture. Sodium oleate was then added in quantity sufficient to kill the diplococci during one hour's contact in the absence of serum. This experiment, which was repeated, showed immediately that other factors besides the calcium entered into the reaction, since the diplococci were protected from the action of the soap by oxalated serum, just as they were by the native serum.

At this point advantage was taken of an observation made by von Liebermann and von Fenyvessy,[38] who noted that the inhibitory effect of serum upon soap hemolysis could be prevented by means of boric acid. The preliminary experiments with boric acid showed that in a 3 per cent. solution this chemical, when acting for the period of one hour, exhibited no appreciable antiseptic or

[38] *Ztschr. f. Immunitätsforsch.*, 1909, ii, 436.

destructive action on pneumococci. It could now be shown that when boric acid was added to the serum in a suitable quantity, it counteracted almost completely the inhibitory effects of the serum. The optimum quantity of the boric acid for the quantities of 0.2 cubic centimeter of serum and one milligram of sodium oleate proved to be from thirty to forty milligrams. When less than thirty milligrams was employed, the inhibition was imperfect; when more than forty milligrams was used, the boric acid itself deterred the action of the soap, and its effect on the serum inhibition became obscured.

Two-tenths cubic centimeter of serum, three milligrams of boric acid, and a quantity of sodium oleate yielding a dilution of 1 to 1,000 are allowed to act upon the pneumococci; it is found that all of those exposed have been killed, and nearly all dissolved. When less than thirty milligrams of boric acid are employed, a part only of the pneumococci are killed and dissolved, while if a greater amount be used, all of the cocci are killed, but few or none are dissolved.

EXPERIMENTS 10.14 AND 10.15. February 24, 25, 1910. Materials used: a twenty-four hour plain bouillon culture of "N. 1.21" made from the heart's blood of a "passage" mouse. 0.000001 c.c. fatal for a mouse. Freshly prepared 0.5 per cent. solution of sodium oleate. 5 per cent. solution of boric acid. Normal goat serum. The various mixtures are made in test tubes and allowed to remain for one hour. Then microscopical preparations and transplantations to glucose serum agar are made. Afterwards a part of each mixture is injected into the peritoneal cavity of a mouse so that each mouse receives 0.5 c.c. of culture, as shown in Table II.

Next, it was observed that the substitution of immune antipneumococcus serum for the normal serum favors the destruction of the diplococci and consequently the recovery of the inoculated animals. The difference in effects between the normal and the immune sera in these experiments is less striking than in the experiments of Table I, for the reason that here the concentration of soap used suffices in itself in the absence of serum to destroy the bacteria, and thus masks the effects of the immune serum. On the other hand, the greater efficiency of the immune over normal serum is shown in two distinct ways: first, by the smaller quantity of boric acid required to produce a given result with immune than with

TABLE II.

		Microscopical preparations.	Transplantations.	Result.
10.62	Culture + soap solution; dilution, 1–1,000.	Outlines of cocci lost; small chromatin-staining masses remain.	Sterile.	Recovered.
10.72	Serum + culture + soap.	No reduction; no morphological change.	Profuse growth.	Died, 17 hrs.
10.74	Boric acid (10 mg.) + serum + culture + soap.	Marked reduction in number; all altered.	Profuse growth.	Died, 18 hrs.
10.75	Boric acid (20 mg.) + serum + culture + soap.	More reduction; all altered.	Profuse growth.	Died, 30 hrs.
10.78	Boric acid (30 mg.) + serum + culture + soap.	Very few remain.	3 colonies.	Recovered.
10.79	Boric acid (40 mg.) + serum + culture + soap.	Only an occasional pair remains.	1 colony after 48 hrs.	Recovered.
10.80	Boric acid (60 mg.) + serum + culture + soap.	Slight reduction in number; some swollen.	Sterile.	Died, 48 hrs.
10.76	Boric acid (60 mg.) + culture.	No change.	Profuse growth.	Died, 16 hrs.
10.73	Boric acid (20 mg.) + culture + soap.	Few pairs left; all altered.	Sterile.	Recovered.
10.77	Boric acid (60 mg.) + culture + soap.	Little or no reduction; some swollen.	Sterile.	Died, 67 hrs.

All the animals that died, except Nos. 10.80 and 10.77, presented at autopsy the usual findings of death from pneumococcic septicemia. Nos. 10.80 and 10.77 died from the toxicity of the substances which were injected, and not from infection. In each there was a barely perceptible, thin peritoneal exudate, which was sterile, as was the heart's blood.

normal serum; and second, by the smaller quantity of immune than of normal serum required to protect the animals from infection. The former fact is brought out by the experiment summarized in Table III.

Experiment 10.16. February 28, 1910. The experiment is carried out in the same manner as experiments 10.14 and 10.15, except that immune goat serum is substituted for the normal serum.

TABLE III.

		Microscopical preparations.	Transplantations.	Result.
10.83	Boric acid (10 mg.) + immune serum + culture + soap.	Marked reduction in number of cocci; all altered.	Profuse growth.	Recovered.
10.84	Boric acid (20 mg.) + immune serum + culture + soap.	More reduction.	Scant growth.	Recovered.
10.85	Boric acid (30 mg.) + immune serum + culture + soap.	Only a few cocci remain.	1 colony.	Recovered.
10.86	Boric acid (40 mg.) + immune serum + culture + soap.	An occasional pair seen.	Sterile.	Recovered.
10.82	Control: boric acid (60 mg.) + immune serum + culture.	No change.	Profuse growth.	Died, 17 hrs.

10.82: autopsy showed usual findings of pneumococcic septicemia.

In this experiment the immunity principles of the serum have sufficed to compensate for the only partial removal of the serum inhibition which was afforded by the insufficient quantities of boric acid, for although viable diplococci were still present in mixtures 10.83 and 10.84, yet the injected animals recovered, while the corresponding ones, 10.74 and 10.75 of the preceding experiment with normal serum, succumbed.

The second way in which the superiority of the immune serum is exhibited is illustrated by the experiment summarized in Table IV, from which it will be seen that smaller quantities of immune serum accomplish what it is impossible for even larger quantities of normal serum to do. In order to keep the conditions of the experiment identical throughout and to secure an equal quantity of protein, normal serum was added to the immune serum to make the quantities of equal volume throughout, namely, 0.2 cubic centimeter, which is the amount required for complete inhibition of the action of the soap.

TABLE IV.

		Transplantations.	Result.
10.94	(Same as 10.74). Boric acid (10 mg.) + normal serum (0.2 c.c.) + culture + soap.	Profuse growth.	Died, 40 hrs.
10.95	(Same as 10.75). Boric acid (20 mg.) + normal serum (0.2 c.c.) + culture + soap.	Moderate growth.	Died, 16 hrs.
10.96	Boric acid (10 mg.) + immune serum (0.05 c.c.) + normal serum (0.15 c.c.) + culture + soap.	Moderate growth.	Recovered.
10.97	Boric acid (20 mg.) + immune serum (0.05 c.c.) + normal serum (0.15 c.c.) + culture + soap.	Moderate growth.	Recovered.
10.98	Boric acid (10 mg.) + immune serum (0.02 c.c.) + normal serum (0.18 c.c.) + culture + soap.	Profuse growth.	Died, 40 hrs.
10.99	Boric acid (20 mg.) + immune serum (0.02 c.c.) + normal serum (0.18 c.c.) + culture + soap.	Slight growth.	Died, 40 hrs.
10.100	Boric acid (10 mg.) + immune serum (0.01 c.c.) + normal serum (0.19 c.c.) + culture + soap.	Profuse growth.	Died, 16 hrs.
10.101	Boric acid (20 mg.) + immune serum (0.01 c.c.) + normal serum (0.19 c.c.) + culture + soap.	Slight growth.	Died, 48 hrs.

From the preceding experiment it is apparent that as little as 0.05 cubic centimeter of the immune serum suffices to prevent infection and death even when as little as from ten to twenty milligrams of

boric acid are used in combination; while the larger quantity, namely, 0.2 cubic centimeter, of normal serum under the same conditions exerts little or no restraining action on the growth and infectivity of the pneumococci.

THERAPEUTIC TESTS WITH SERUM, SOAP, AND BORIC ACID MIXTURES.

Now that it has been ascertained that boric acid is capable of preventing the protein inhibition of soap *in vitro,* the question at once presents itself as to whether it will exercise a similar effect *intra vitam,* and if so, whether use can be made of soap and immune serum mixtures to prevent infections with the pneumococcus. With respect to this question it can be stated that rats which have received intraperitoneal injections of several times the fatal dose of a virulent culture of pneumococci may be saved from fatal infection by the subsequent injection of an appropriate mixture of immune serum, boric acid, and sodium oleate. In brief, it has been found that when what we shall call the therapeutic injection of the mixture described above is made to follow immediately the inoculation of the pneumococci or is made within one hour of the inoculation of the organisms, all of the animals so treated survive. When the treatment is postponed longer, so that the interval between the inoculation and the treatment is as long as two hours, about one-half of the treated animals survive, and when the treatment is still further postponed, the results are less satisfactory.

The experiment summarized in Table V is taken from a series, all the experiments of which resulted similarly.

EXPERIMENT 10.28. April 25, 1910. Materials used: a twenty-four hour plain broth culture of "N. 1.78" made from the heart's blood of a "passage" mouse; 0.00001 c.c. fatal for a rat. A mixture, each cubic centimeter of which contains 0.2 c.c. of immune goat serum, 0.6 c.c. of a 5 per cent. solution of boric acid, and 0.2 c.c. of a 0.5 per cent. solution of sodium oleate.

The experiment was performed in duplicate, eight white rats being used in each series. The culture was injected first and then the therapeutic mixture, as shown in Table V.

The interpretation of the results of the therapeutic experiments appears to be as follows:

TABLE V.

10.153	10.161	Control : 0.01 c.c. culture.	Both died, about 40 hrs.
10.154	10.162	Control : 0.1 c.c. culture.	10.154 died, about 18 hrs.; 10.162 died, about 40 hrs.
10.155	10.163	0.01 c.c. culture + 0.5 c.c. therapeutic mixture immediately.	Both recovered.
10.156	10.164	0.1 c.c. culture + 0.5 c.c. therapeutic mixture immediately.	Both recovered.
10.157	10.165	0.01 c.c. culture + 0.5 c.c. therapeutic mixture 2 hrs. later.	10.157 recovered ; 10.165 died, 3½ days.
10.158	10.166	0.1 c.c. culture + 0.5 c.c. therapeutic mixture 2 hrs. later.	10.158 died, 4½ days ; 10.166 recovered.
10.159	10.167	0.01 c.c. culture + 0.5 c.c. therapeutic mixture 2 hrs. and 24 hrs. later.	Both recovered.
10.160	10.168	0.1 c.c. culture + 0.5 c.c. therapeutic mixture 2 hrs. and 24 hrs. later.	10.160 recovered ; 10.168 died, 5 days.

Although rat 10.165 had, at autopsy, a pneumococcic peritonitis and septicemia, its right lung was the seat of an extensive pseudo-tuberculosis, which is common in laboratory rats, and which may have contributed to its death.

In inhibiting the union of sodium oleate with the serum proteins, the action of boric acid is not perfect. It depends in part upon the amount of protein present and is greatest only under definite conditions. Hence, its effects *intra vitam* are greater during a period in which the protein of the serum exuded into the peritoneal cavity is not excessive, and its action becomes less perfect, and hence inadequate, when the exudate has become rich in serum protein. Moreover, the degree of multiplication of the pneumococci must also be taken into account since the action of the therapeutic mixtures upon the virulent diplococci is not indefinite; and in the experiments, multiples of the fatal dose having been inoculated, account must be taken of the multiplication occurring within the first two hours following the infection, which multiplication adds to the number of fatal doses present. Even the forming exudate in the peritoneal cavity is, by nature of the anatomical relations there present, likely to be enclosed by adhesions in pockets to which the therapeutic mixture may not have free access. Finally, the rat is, under the circumstances of the experiment, a species highly susceptible to infection, and particularly to septicemia; and within the period of two hours the pneumococci injected into the peritoneal cavity have gained access to the blood and through the blood to the internal organs, in which situations they are not readily, if at all, reached by the therapeutic mixture.

The problem presented in the rescue of these animals from a fatal infection is a severe one as compared with that presented by local affections due to the pneumococcus occurring in larger animals subject to spontaneous infection.

MECHANISM OF THE PROCESS.

The precise mechanism of the combined action of sodium oleate and immune serum, through which the growth of the pneumococci is so greatly restrained and their solution so much promoted, should be discussed. That the dilute solutions of the alkaline oleates produce definite and marked changes in the organisms that do not directly affect their viability is obvious. These changes are profound enough to bring the viable pneumococci within the dissolving sphere of serum, from which it would appear that one effect, at least, of the sodium oleate is to render the organisms more pervious for the serum. We wish to suggest that this action of the soap is connected in some degree with the lipoidal constituent of the organisms and possibly with the moiety contained within their outer membranes which, under the influence of the soap, having become attenuated or partly dissolved, permit ingress of the serum. To this conception of the manner of action of the soap would conform the greater tendency of the soaped pneumococci to undergo autolysis, since the weakening of the lipoidal barrier would favor the action, in the manner described by Hans Meyer,[39] of the autolytic ferments upon the bacterial cell. The bacterial cells thus altered by the action of the soap become apparently more pervious to an immune than to a normal serum, doubtless because of the presence in the former of principles, possibly of colloidal nature, which favor its entrance into the bacterial cell. Doubtless, also, the specific immunity principle exercises directly a destructive action upon the pneumococci, for the completion of which complement would seem not to be required.

Further experiment is required in order to ascertain to what extent the peculiar action of the alkaline soaps described is applicable to other members of the group of the pyogenic cocci. In this connection it should be recalled that the pneumococcus has already been

[39] *München. med. Wchnschr.*, 1909, lvi, 1577.

distinguished from other pyogenic cocci by reason of the fact that it is subject to solution by bile, while the others are not. Studies are in progress for the purpose of determining the manner of reaction of other members of the pyogenic bacterial group to the alkaline soaps.

CONCLUSIONS.

Highly dilute solutions of the alkaline oleates, which do not suffice to alter appreciably the morphology or reproductive power of the pneumococci, nevertheless produce profound changes in their structure.

Pneumococci treated with sodium oleate become more subject to autolysis, as is indicated both by the rapidity and the perfection of the process of self-digestion, and at the same time they become subject to serum-lysis.

The serum-lysis of the soaped pneumococci tends to be incomplete with normal serum and to be perfect with an immune anti-pneumococcus serum. When normal serum is employed, the surviving pneumococci subsequently multiply either in the test tube or in the animal body, in the latter case producing fatal infection. When, on the other hand, an immune serum is employed, lysis is complete, no multiplication occurs, the test tube mixture is sterilized, and the inoculated animal is protected from infection.

The inhibition of their activity which the soaps ordinarily suffer in the presence of protein, can be prevented by the addition of an appropriate quantity of boric acid, so that suitable mixtures of serum, soap, and boric acid can continue to exert a deleterious and solvent influence on the pneumococci, and the effect is greater when immune serum is employed in the mixtures.

Infection can not only be prevented when the mixture of immune serum, soap, and boric acid is added to the pneumococci before injection into the peritoneal cavity of small animals, but the infection can likewise be prevented when a therapeutic injection of a mixture of the three substances mentioned is made to follow the inoculation of normal, highly virulent diplococci. The limits of the activity of the therapeutic mixture are determined, in part by the amount of protein to be overcome, and in part by the peculiarities of the infection occurring in highly susceptible animal species.

The virulence of the pneumococci is somewhat diminished by the soap treatment, but the treated organisms are not rendered more subject to phagocytosis.

It would appear that the action of the soap is exerted upon the lipoidal moiety of the bacterial cells, through which they are rendered more pervious to serum constituents and brought under their deleterious and dissolving influence. The changes in the pneumococci here described probably have a prototype in the resolving exudate of a pneumonic process, so that it may be considered that they occur in the animal body in the course of spontaneous infection and constitute one of the conditions of the conquest of the organism by the body's forces. A certain conformity exists between the manner of destruction of the pneumococci in a pneumonic exudate and that in the artificially prepared soap-serum mixtures.

In order to imitate outside of the body the conditions of the removal of pneumococci within the body, it does not suffice merely to study the reactions to leucocytes and serum and to conclude from these reactions the means which the body employs for the disposal of the diplococci; but it is necessary to invoke still other factors, among which are the effects of chemical substances present in exudates, of which the soaps represent one class. The failure hitherto to unify the reactions in test tubes with those occurring in the body in connection with the pneumococcus may be due to the fact that account was not taken of this class of chemical bodies.

Whether the principle here presented can be made applicable to the treatment of local pneumococcus infections in human beings is a pressing question. Its application to the treatment of local infections, to the seats of which the serum, soap, and boric acid mixtures can be directly applied, particularly after evacuation of an inflammatory exudate, seems to offer promise.

ON ARTERIOVENOUS ANASTOMOSIS AND THE MECHANICAL THEORY OF ARTERIOSCLEROSIS.[1]

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Plates I–IV.

The function of a blood-vessel is purely mechanical and consists either in lessening the strength of the onrushing blood sent by the systole of the heart, or in assisting the blood-flow, the strength of which is diminished as it nears the periphery. Both of these functions are taken up by the elastic and muscular fibers of the media. In view of this, it is evident *a priori* that the mechanical conditions of the blood stream within the lumen of the vessels must play a certain rôle in the causation of arteriosclerosis. Nevertheless, this factor was completely lost sight of until the appearance of the first publication of Thoma (1) in 1883. This investigator was the first to indicate the importance of the increase in blood pressure on the etiology of diseases of the blood-vessels.

The wall of a blood-vessel, weakened by a degenerative process, presents little resistance to blood pressure and, as a consequence, the lesion is continually increased. But Thoma and his adherents go a step further and claim that when an artery has no preliminary degeneration of its media, a lesion of the blood-vessel wall will ensue if the blood pressure is above normal.

The proliferation of the endothelial cells of the intima, which is an early concomitant of the degenerative process of the media, is explained by these investigators by a compensatory filling-in of the depression in the wall, caused by the thinning of the diseased part of the media.

Thoma adduced the following experimental proof of this contention: when a diseased aorta is filled with melted lard under pres-

[1] Read before The American Association of Pathologists and Bacteriologists, Washington, D.C., May, 1910. Received for publication, June 6, 1910.

sure, the projecting intimal nodosities disappear. Thus, a plausible explanation of the main characteristics of arteriosclerosis is offered on the basis of the mechanical theory. But while the deleterious effect of blood pressure on a previously diseased vessel wall may be self-evident, the possibility of producing arteriosclerosis by increased pressure alone needs experimental proof.

Thoma tried to approach the subject experimentally by producing in dogs an insufficiency of the aortic valve, and he claims to have observed subsequently arterial lesions. These were not identical with arteriosclerosis, consisting merely in hypertrophy of the intima. His results could not be confirmed either by Poper (2) or Jores (3).

Another experimental method was made use of, apparently with great success, by Josué, who reported, in 1903, that he had artificially produced "atheroma" in the aorta of rabbits by intravenous injections of adrenalin. Josué considered his results as an experimental proof of the mechanical theory of arteriosclerosis, since the action of adrenalin consists in raising blood pressure. Identical results have since been obtained by a great many investigators, but it is doubtful whether these results can be used in support of Thoma's theory. In the first place, spontaneous arterial lesions are found so frequently in the rabbit, that this animal does not appear to be suitable for the study of experimental arteriosclerosis. Indeed, our recent investigations (4) have shown that while only 13 per cent. of 240 rabbits showed an arterial lesion in the aorta on gross inspection, 39 per cent. of the other rabbits showed also spontaneous lesions, though in such an early stage that they could be detected only on microscopical examination. In other words, 52 per cent. of the supposedly normal rabbits had diseased blood-vessels, and this is nearly the same percentage as is usually found after treatment with adrenalin.

The microphotographs shown in Plate I, Figs. 1, 2, and 3, and Plate II, Figs. 4, 5, and 6, show that the minute spontaneous lesions are identical with the lesions found after treating rabbits with adrenalin. Furthermore, the possibility cannot be excluded that adrenalin, which is a powerful poison, may not have, besides its blood pressure-raising influence, certain toxic effects upon the organism and, consequently, upon the wall of the blood-vessel.

The same lesions were also induced in rabbits by a great many substances; some raised the blood pressure, others lowered it, and others still, like the bacterial toxins, had no effect whatever on the blood pressure.

On the other hand, all attempts to produce arterial lesions in normal dogs or other laboratory animals by injection of adrenalin failed. The following question then presents itself: is it possible to produce in dogs, by other mechanical means, conditions similar to arteriosclerosis? Carrel and Guthrie (5) have shown that the walls of a segment of a vein implanted in an artery, undergo certain changes which consist in a hyperplasia of the connective tissue of the adventitia and an increase of the number of the muscular and elastic fibers of the media. On such an implantation Stich (6) found conditions which he considers similar to arteriosclerosis. Fischer and Schmieden (7) found a slight hypertrophy of the media with an increased number of connective tissue fibers and a rather diminished number of elastic tissue fibers. The intima was thickened only in the neighborhood of the suture.

It is hardly possible to draw correct conclusions from this method of experimentation, since such a segment is completely severed from its vascular connection and its cellular elements are placed under unfavorable conditions of nutrition. Our recent investigations (8) lead us to suppose that such an implanted segment may ultimately become absorbed, and that a complete new tube may form around the skeleton of the implanted segment.

The conditions are nearer normal when a unilateral anastomosis is made, *i. e.*, when two vessels are united without severing the connection of either with the general circulation. Carrel and Guthrie performed the unilateral anastomosis and found that the wall of the artery contained an increased amount of connective tissue. Fischer and Schmieden, also, performed this operation twice, but they did not keep the dogs long enough to come to any definite conclusion.

In our experiments we endeavored to increase the blood pressure of a vein by anastomosing to its central end the peripheral end of an artery, thus not only preserving the circulation within the vein, but also adding to it the pressure of the arterial blood flowing from the artery. A certain number of the dogs received three to five intrave-

nous injections of adrenalin, in order to determine whether an effect could be seen in a vein already placed under unfavorable conditions of pressure. Previous to this, on two dogs a side-to-side anastomosis of the aorta and vena cava was tried, with the hope of increasing the pressure in the latter blood-vessel, but in both experiments great congestion of all the parenchymatous organs took place, and the animals died within twenty-four hours. Then, on four dogs side-to-side anastomosis was tried between the femoral artery and vein. The animals were killed two to three months later; both the artery and vein were found to be patent and the circulation perfect, but the opening made between the two vessels was completely healed and covered with endothelium.

It was then decided to resort to end-to-end anastomosis between the external carotid and the external jugular. Ten experiments were performed; a description of each is given below.

EXPERIMENT 1.—November 13, 1909. Brown female dog: anastomosis between right external carotid and external jugular.

January 5, 1910. The second operation was performed on the same animal. Anastomosis between left external carotid and external jugular.

During and before the second operation, venous pulsation could be felt in the right external jugular. The second operative field became infected; a large abscess formed, which gradually healed, leaving a suppurating sinus.

February 1. Animal killed. At the post-mortem examination the right carotid and jugular appeared thrombosed for about two and one-half inches; very fine wire could be passed through the lumen. Tissue was hard, elastic and cord-like. Microscopical examination showed growth of connective tissue in the intermediary coat of the intima and growing into the lumen of the vessel.

EXPERIMENT 2.—December 12, 1909. White and black male dog: anastomosis between right external carotid and external jugular.

January 19, 1910. The animal received an intravenous injection of 8 mm. of adrenalin.

January 23, 27, and 29. The same injection was repeated.

March 24. The animal was killed. Post-mortem examination showed a fusiform aneurismal dilatation of the vein. On gross inspection the vessel wall looked slightly thickened, but there was no evidence of a change in the endothelial lining. Microscopical examination shows no abnormality either in the intima or media of the vein. There is fibrous tissue hyperplasia in the adventitia of the vessel.

EXPERIMENT 3.—February 2, 1910. Brown female dog: anastomosis between right external carotid and external jugular. No adrenalin treatment; dog was killed March 12. Post-mortem findings the same as in experiment 2, only the vessel wall of the vein appears somewhat thicker than the vein of dog 2. Microscopical appearance was the same as in dog 2.

EXPERIMENT 4.—February 9, 1910. Brown female dog: anastomosis between right external carotid and external jugular. The dog received four injections of adrenalin. Killed April 22. Post-mortem findings were the same as in experiment 2.

EXPERIMENT 5.—February 6, 1910. Black male dog: anastomosis between right external carotid and external jugular. Four injections of adrenalin. Dog killed April 14. Post-mortem examination shows a moderate distention of the external jugular vein at its distal part. The artery and vein for a distance of half an inch on each side of the line of suture is firm, thickened, and elastic. A fine wire probe passes through the contracted lumen. Microscopical examination shows parietal organized thrombus. Connective tissue hyperplasia in the adventitia. Distal section of vein is normal.

EXPERIMENT 6.—January 19, 1910. Large, brown female dog: anastomosis between right external carotid and external jugular. Five injections of adrenalin. Killed April 7. Post-mortem examination shows normal jugular vein; no distention or thickening of the wall.

EXPERIMENT 7.—March 23, 1910. Small, brown female dog: anastomosis between right external carotid and external jugular. Three injections of adrenalin. Dog killed April 7. Post-mortem findings were the same as in experiment 6.

EXPERIMENT 8.—December 4, 1909. Black female dog: anastomosis between right external carotid and external jugular. Four injections of adrenalin. Dog killed April 7. Post-mortem examination shows aneurismal dilatation of vein for about half a inch above anastomosis. Beyond this point the vein is not markedly dilated. Dissection of the artery and vein shows aneurismal dilatation in vein to be due to mechanical impact of arterial blood against the wall of the vein. No abnormality in the intima or media is seen on microscopical examination.

EXPERIMENT 9.—February 23, 1910. Medium-sized, white male dog; anastomosis between the right external carotid and external jugular. No adrenalin injection. April 22, dog killed. The result of the post-mortem examination was the same as in dog 8.

EXPERIMENT 10.—March 9, 1910. Light, bushy, female dog: anastomosis between the right external carotid and external jugular. Four injections of adrenalin. Dog killed April 22. Post-mortem findings were the same as in experiment 8.

Analysis of these experiments shows that not in a single instance did the vessel wall of the external jugular undergo the degenerative process characteristic of arteriosclerosis, though the pressure exerted on its wall must have been considerable, since at one end there was a stream of arterial blood from the carotid, and at the other end the normal venous circulation pressing against the carotid. Moreover, in several instances the animal received from three to five injections of adrenalin. The dilatation that was found

in six out of the ten experiments is not identical with the usual condition of aneurism, since the vessel wall does not show any degeneration and retains its elastic lamina (Plate III, Figs. 7 and 8). Only at the level of the suture was there a noticeable hyperplasia of the intima. This was apparently due to the irritation caused by the silk suture acting as a foreign body. The thickening of the walls of veins, found occasionally on gross inspection, is due to connective tissue proliferation of the adventitia caused by the stripping off of the adventitia during the operation (Plate IV, Fig. 9). The obliterative endarteritis found in experiment 1 is due to an infection which took place after the second operation on the same dog, and in experiment 5 it occurred subsequently to the primary operation.

Consequently, as a result of our investigation, the conclusion must be drawn that arteriosclerosis cannot be artificially induced in a previously healthy blood-vessel by a change in the blood pressure alone, and that the thickening of the wall and the other changes noticed in such vessels is probably due to changes in the adventitia caused by the operative procedures.

EXPLANATION OF PLATES.

Plate I.

Fig. 1. Aorta of normal rabbit. Hematoxylin-eosin, low-power: small area of degeneration in media reaching intima. Lime deposits.

Fig. 2. Aorta of normal rabbit. Hematoxylin-eosin, high-power: small area of degeneration in media reaching intima. Lime deposits.

Fig. 3. Aorta of normal rabbit. Hematoxylin-eosin: two areas of degeneration in media. Lime deposits.

Plate II.

Fig. 4. Aorta of normal rabbit. Weigert's elastic tissue stain: two areas of degeneration in media. Same specimen as Fig. 3.

Fig. 5. Aorta of normal rabbit. Hematoxylin-eosin, low-power; minute nodule in the intima.

Fig. 6. Aorta of normal rabbit. Weigert's elastic stain: minute nodule of the intima shows no elastic tissue.

Plate III.

Fig. 7. Anastomosis between external carotid and external jugular. The two figures to the left show dilatation of the vein split open. The third specimen shows that the dilatation of the vein reaches only above the valve. The fourth specimen is the same as the first before opening.

FIG. 8. Dog 3. Anastomosis between carotid and jugular. Hematoxylin-eosin, low-power, longitudinal section: in the middle of the section is the line of anastomosis; artery to left, vein to right.

PLATE IV.

FIG. 9. Dog 3. Anastomosis between carotid and jugular vein. Hematoxylin-eosin: connective tissue proliferation in adventitia.

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FIG. 1.

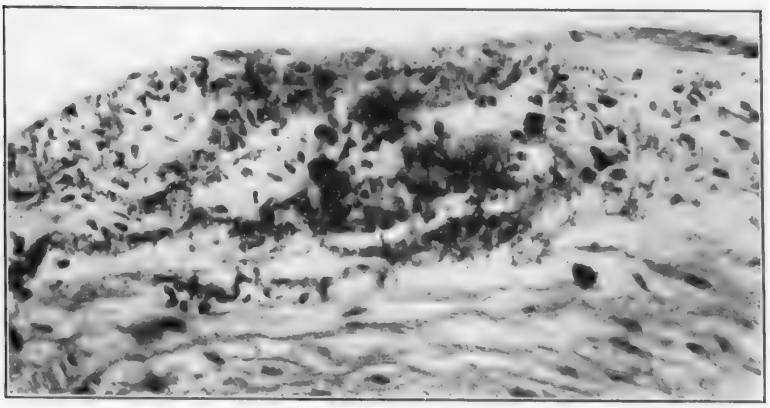
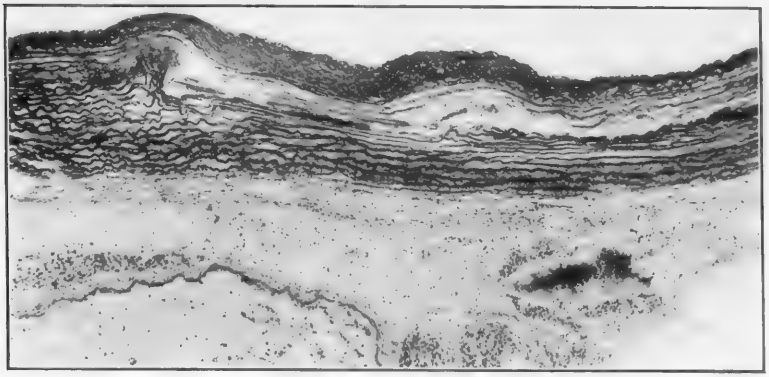


FIG. 2.

FIG. 3.

Mail



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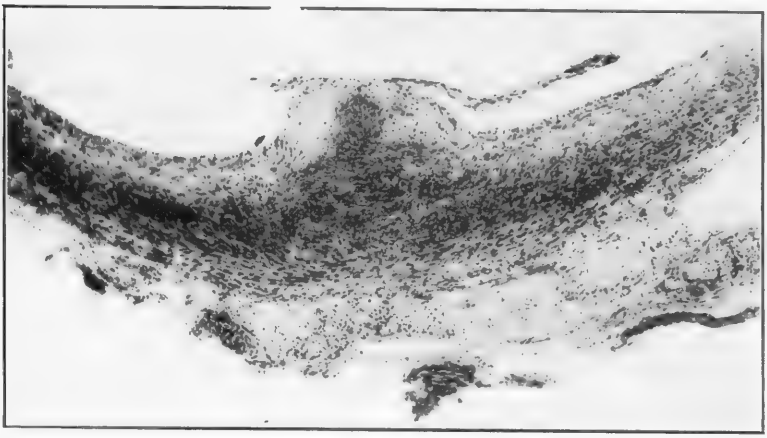

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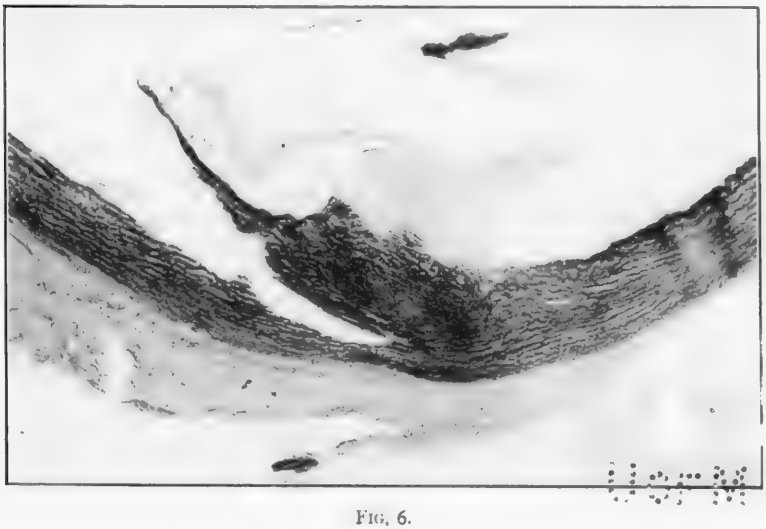

FIG. 6.

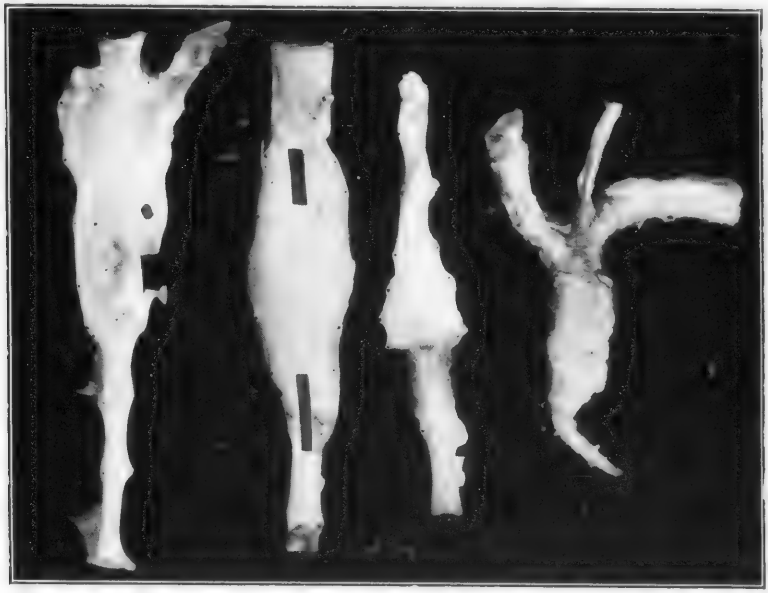

FIG. 7.

FIG. 8.

FIG. 9.

ON HEAT-SENSITIVE ANTICOMPLEMENTARY BODIES IN HUMAN BLOOD SERUM.[1]

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INTRODUCTION.

It has long been well known that normal human and animal blood sera may, under certain conditions, contain elements which exert antihemolytic activity when added to mixtures of red blood cells, hemolytic serum (amboceptor), and complement. The work of most of the writers who have studied these bodies has revealed that normal sera, showing no evidence of antihemolytic action originally, may develop this property when subjected to heat ranging from 50° to 56° C. Such has been the experience of P. Th. Müller (1), Ehrlich and Sachs (3), Noguchi (8), and others. Once formed, moreover, this class of antihemolysins has been found, by some workers, to possess no inconsiderable degree of thermostability, Noguchi finding that 90° C. were necessary to destroy completely the inhibitory properties of such sera.

There is still another class of antihemolytic substances, however, which may appear in normal sera that have not been subjected to heat. Müller mentions the occurrence of such bodies in rabbit and guinea-pig sera studied by him, and attributes their inhibiting properties to direct anticomplementary action. Noguchi, who has studied them in greater detail in connection with his recent work on the "Serum Diagnosis of Syphilis," states that such bodies are not present in fresh human serum, but develop gradually as the serum is preserved, and may be eliminated by heating to a temperature of 54° C. for thirty minutes.

There appear to be two distinct antihemolytic bodies, therefore, which may occur in normal sera. One of these is found in sera after heating, and is relatively thermostable. The other develops in sera on standing, and is destroyed by temperatures which are ordinarily employed for serum inactivation, 54° to 56° C.

The development of specific antibacteriolytic and antihemolytic substances in immune sera previously treated with bacteria or red blood cells, respectively, as described by Pfeiffer and Friedberger (7), and later by Sachs, may, we believe, be ignored, since Gay (5) has satisfactorily shown that the phenomena observed by Sachs did not occur when the corpuscles originally added to the serum had been sufficiently washed. Gay showed that the antihemolytic action observed by Sachs was due to complement absorption by means of specific precipitates formed between the immune serum and the alien serum carried into the mixtures with the insufficiently washed corpuscles.

The thermostable antihemolytic bodies referred to above have been extensively studied. That their inhibitory action depends upon a directly anticomplementary

[1] Received for publication, September 6, 1910.

mechanism, all workers agree. As to the nature of the substances themselves, however, there is much discrepancy of opinion. Müller believed that the antilytic elements were probably present in the native serum but were temporarily masked by hemolytic elements simultaneously present. When the action of the latter had been eliminated by heating, the action of the former became evident.

Ehrlich and Sachs suggest that the application of heat serves, in some cases at least (dog serum heated to 51° C.), to transform the complement into a complementoid with a higher avidity for sensitized corpuscles than that possessed by the complement itself.

Sachs, who attributed the anticomplementary action to complement deviation by free amboceptors of many varieties, assumed to be present in the serum, has apparently been conclusively answered by Gay. His view, moreover, is opposed to present experience, which seems to indicate that complement is not bound by amboceptor uncombined with its specific antigen.

Noguchi, whose studies of the thermostable antihemolysins of heated sera have been extensive, has come to the conclusion that these bodies are set free in sera as the latter are subjected to heat, more and more being liberated as the temperature is raised above 50° C. At 90° C. he believes that these bodies are either inactivated by conversion into more stable compounds or are masked by the formation of new bodies, "auxilysins," which aid hemolysis. He finds that the inhibitory substances are anticomplementary, active against alien as well as against homologous complement, and that they may be removed from the serum by digestion with red blood corpuscles, the corpuscles becoming thereby more resistant to hemolytic influence. Finally, he states that the antihemolysin, which he names "protectin," may be obtained from such sera as lipoids, or together with these, by ether extraction.

The antihemolysins occurring in unheated sera have been less extensively studied, and it was these bodies particularly to which the attention of the writers was directed.

Occurrence of the Thermolabile Antihemolytic Substances.—The development of antihemolytic substances in unheated human blood sera was noticed by the writers, as it had been by Noguchi, in the course of work with complement-fixation tests. It was observed, also, that the inhibitory bodies did not occur in fresh, active sera, but developed as the sera were preserved at room or refrigerator temperatures, and that, as stated by Noguchi, heating to 56° C. for fifteen to thirty minutes served to eliminate the antilytic powers. After a number of such sera had been studied, it was deemed desirable to ascertain the speed of development of these bodies in normal human serum (animal sera were not investigated), and a series of sixteen normal specimens were examined for their inhibitory powers at intervals of three to five days.

In these experiments .2 cubic centimeter of the serum to be examined was added to .1 cubic centimeter of fresh guinea-pig complement, the volume was brought up to 3 centimeters with salt solution, and the mixture was allowed to stand in a water bath at 40° C. for thirty minutes. At the end of this time 1 cubic centimeter of a 5 per cent. emulsion of red blood cells sensitized with two units of amboceptor were added, the total volume being brought to 5 cubic centimeters. The usual controls were made.

By such experiments it was found that some sera, kept at refrigerator temperature (about 10° C.), would become distinctly antilytic as early as the fifth day, and many completely inhibited hemolysis at the end of two weeks. In the case of two sera not in this particular series, complete inhibitory power, for the quantities given, appeared as early as the fifth day. Other sera exhibited no trace of antihemolytic power after four and five weeks. These sera were examined at the same time for complement, and in every case where no antihemolytic substances had developed, free complement could be detected.

It is probable that these thermolabile bodies develop in most, if not in all, human sera on standing, appearing as the complement disappears, and that they are originally either not present or are masked in their action as long as traces of complement remain.

Two of the sera in which the antihemolytic power developed most rapidly and strongly were taken from patients suffering from chronic nephritis with symptoms of uremia. As this seemed significant, in view of the paper of Neisser and Döring (2), who have claimed the presence of antihemolytic substances in uremic sera, a number of tests were made with sera from similar cases and from two cases of eclampsia. No evidence was obtained, however, which justified any conclusion regarding a definite relationship between the clinical condition and these substances.

In an experiment to determine the temperature at which the inhibitory action was eliminated, sera (after dilution)[2] were heated to 90° C. It was found that most of the antihemolytic action (roughly speaking, about four-fifths) had disappeared. The fact

[2] In all our experiments the volumes of the mixtures were brought to five cubic centimeters with salt solution to avoid errors resulting from the inhibitory influence of high concentrations of serum, which, as shown by Bordet and Gay (10), oppose hemolysis.

that a trace still remained would tend to show either that the thermolabile and thermostabile antilytic bodies may be present at the same time in the same serum or that the latter may be developed in a serum that contained the former.

Quantitative Titration of the Thermolabile Antihemolytic Body in Some Sera.—The strength of the antihemolytic power developed in some of the human sera, may be seen in the following titration, in which the serum employed was about three weeks old:

Complement 0.1 c.c., Corpuscles (5 per cent. emulsion) 1.0 c.c., 2 units amboceptor.	+	0.2	c.c. serum "W"	No hemolysis.
		0.1	c.c. serum "W"	No hemolysis.
		0.05	c.c. serum "W"	Very slight hemolysis.
		0.04	c.c. serum "W"	Slight hemolysis.
		0.03	c.c. serum "W"	
		0.02	c.c. serum "W"	
		0.01	c.c. serum "W"	Considerable hemolysis, but incomplete.
		0.005	c.c. serum "W"	
		0.0025	c.c. serum "W"	Hemolysis complete.
		0.00125	c.c. serum "W"	Hemolysis complete.

It will be seen, therefore, that quantities as small as 0.005 cubic centimeter of this serum exerted considerable inhibiting action.

Serum "W," employed in the above experiment, had the strongest antihemolytic action of any in our possession. As early as the fifth day, 0.1 cubic centimeter of it sufficed to prevent completely the hemolysis of one cubic centimeter of sensitized corpuscles.

Does the Antihemolytic Action Bear Any Relationship to Substances Derived from the Red Corpuscles?—Since a number of the sera which showed antilytic properties were observed to be tinged with blood pigment, owing to accidents during separation, one of the thoughts first suggested was that the antilytic bodies might be derivatives of red blood cells, given up to the serum during its separation from the clot. This seemed the more likely because of the observations of Landsteiner and von Eisler (4), Muir (9), and others, that certain red cell extractives did actually exert antilytic action. A considerable number of experiments were made by the writers with this point in view. For the sake of brevity, however, these experiments will be omitted. It is sufficient to say that they showed that both the shadows and products of hemolyzed red cells, when added to a hemolytic system, lent slight antilytic action to sera

otherwise not antilytic. These substances exerted, also, a slightly inhibitory action upon hemolysis when added without the presence of human serum. In these respects the experiments confirmed the observations of other writers. In no case, however, did this antilytic action amount to more than a definite slowing of the hemolytic reaction, or, at most, to an extremely slight permanent inhibition. It was impossible, therefore, to attribute the very marked and often complete inhibitory action of some sera, often perfectly clear, to accidental extractions from the clot. For this reason, this course of experimentation was not carried further and is not enlarged upon. It was of use only in again calling attention to the necessity for caution when doing complement fixation tests with sera containing any considerable amount of blood pigment.

MECHANISM OF THE ACTION OF THE THERMOLABILE ANTIHEMOLYTIC BODY.

In speaking of the thermolabile antihemolytic substance, Noguchi stated that its action was anticomplementary. As it seemed to us desirable to verify this, a number of experiments were made.

In one of these a mixture consisting of two units of anti-sheep amboceptor (1/660 cubic centimeter serum in 1 cubic centimeter salt solution), 0.2 cubic centimeter of inhibiting serum "W," and 3 cubic centimeters salt solution, was incubated for one hour at 40° C., and then after the addition of 1 cubic centimeter of a 5 per cent. emulsion of sheep corpuscles, the whole was incubated for one hour at 40° C., and separated by centrifugalization into (1) sediment of red blood cells (subsequently washed three times with salt solution), and (2) supernatant fluid.

To the washed red cell sediment were added 0.1 cubic centimeter of complement and 5 cubic centimeters salt solution. On exposing the mixture to 40° C. for half an hour, the hemolysis was complete. The hemolysis was also complete in the controls. To 3 cubic centimeters of the supernatant fluid (see (2) above) were added 0.1 cubic centimeter complement and 2 cubic centimeters sensitized corpuscles (1 cubic centimeter corpuscles to which two units of amboceptor had been added). On exposing this mixture to 40° C. no hemolysis occurred.

The above and similar experiments showed that the exposure of amboceptor to the inhibiting substance did not prevent amboceptor from becoming attached to the red cells subsequently added. They indicated, furthermore, that exposure of the "sensitized" cells to

this antilytic substance did not deprive these cells of the power of subsequently absorbing complement. Neither the different receptors of the amboceptor, nor the complex of amboceptor and red cell were, therefore, in any way altered by the antilytic body, this remaining active in the supernatant fluid. The possibility of explaining the antilytic action as due to the presence of complementoid, was also disposed of by these experiments.

It remained to show that there was no reaction between the antilytic body and the unsensitized blood corpuscle rendering the latter resistant to the subsequent influence of amboceptor and complement.

To determine this point, 0.2 cubic centimeter of antilytic serum "W," 1 cubic centimeter of washed sheep corpuscles (5 per cent. emulsion in salt solution), and 2 cubic centimeters salt solution were mixed, left for one hour at 40° C., and then centrifugalized. The sediment containing the red cells was washed four times in salt solution and then to it were added one unit of amboceptor, 0.1 cubic centimeter of complement, and 4 cubic centimeters of salt solution. On incubating at 40° C. complete hemolysis took place in half an hour (controls hemolyzed in the same length of time). This showed that the corpuscles had not been rendered less sensitive to lysis by exposure to the inhibiting serum. The supernatant fluid from the centrifugalization, containing the 0.2 cubic centimeter of the antilytic serum, was then tested and found to have lost none of its inhibiting power.

This seemed conclusive in excluding the direct action of the antilytic substance upon the corpuscles. Nevertheless, as Noguchi had found that the thermostabile antilytic bodies could be absorbed by blood corpuscles if allowed to digest with them at 20° C. for about eighteen hours, it seemed desirable to determine whether the thermolabile body studied by us would be similarly absorbed. To test this question, the following experiments were made on two separate occasions, each time in triplicate.

One cubic centimeter of the antilytic serum was mixed with 4 cubic centimeters of a 5 per cent. suspension of washed corpuscles, and this mixture was left at 18° to 20° C., in one case for twenty, in another for twenty-four hours. At the end of this time the fluid and corpuscles were separated by centrifugalization. The corpuscles were then thoroughly washed, and they and the supernatant fluid were tested as in the preceding experiment.

The result showed (1) that the corpuscles had not absorbed the antilytic principle, for they had not become more resistant to lysis, and (2) that the supernatant liquid had retained its antilytic property undiminished.

As we had thus eliminated the action of the antilytic body upon the corpuscles, the amboceptor, or the corpuscle-amboceptor complex, its action upon the complement remained as the only possibility. This has been proved in a considerable number of experiments which consisted in exposing complement to the antilytic serum for varying periods, and then adding sensitized corpuscles. Complete inhibition of hemolysis has been the uniform result of such a procedure. That the anticomplementary action of the body in question is almost instantaneous, or, at least, more rapid than the attachment of complement to the antigen-amboceptor complex, was shown by the fact that complete inhibition resulted when antilytic serum, complement, and sensitized corpuscles were simultaneously poured into a test tube and immediately incubated.

It was also ascertained that this anticomplementary action could be overcome by gradually increasing the relative amount of complement in the mixtures. Thus, 0.2 cubic centimeter of the antilytic serum "W" completely inhibited hemolysis in the presence of 0.2, 0.4 and 0.6 cubic centimeter of fresh guinea-pig complement, when the cells employed were sensitized with two units of amboceptor. Eight-tenths cubic centimeter of complement, however, caused considerable hemolysis in such mixtures, and one cubic centimeter produced almost complete lysis.

The foregoing experiments thus show, in perfect agreement with the statements of Noguchi, that the mechanism of the action of the thermolabile antihemolytic body is an anticomplementary one.

It seemed of interest to make sure that this anticomplementary action was direct, and not indirect, in the sense that it might depend, (*a*) upon the absorption of complement by one or a number of antigen-amboceptor complexes preëxisting in the serum, or (*b*) upon complement absorption by a precipitin reaction occurring between the antilytic serum and either the guinea-pig serum of the complement or the rabbit serum of the amboceptor.

The first supposition, namely, that the complement is deviated or absorbed by antigen-amboceptor complexes preëxistent in the serum, is difficult to approach experimentally. It seems unlikely, however, that this supposition is correct, for in the experiments cited above, in which the antilytic body, sensitized corpus-

cles, and complement, in excess of the minimum quantity for complete lysis, were mixed simultaneously, absolutely no hemolysis took place. It does not seem reasonable to believe that in such a case *all* the complement should immediately be taken up by the preëxistent antigen-antibody complex, leaving none available for the hemolytic system. Furthermore, it must be remembered that the sera containing the antilytic principle were not inactivated and, when fresh, contained some excess complement. If antigen-antibody combinations had been present, they would, in these cases, be already supplied with complement, and their reaction complexes would be complete. Moreover, the thermolability of the inhibitory substances (which become inactive on heating to 56° C.), is also against this view.

The second suggestion, namely, that complement was deviated by precipitin reactions taking place between the antilytic serum, on the one hand, and rabbit serum or guinea-pig serum, on the other, was disproved by the following experiments:

(*a*) A precipitin reaction between the inhibiting serum and the rabbit serum of the amboceptor was excluded by sensitizing corpuscles and subsequently washing them very thoroughly (four times) in salt solution. The subsequent addition of antilytic serum and complement resulted in complete inhibition, showing that after the removal of all traces of rabbit serum no diminution of the anticomplementary phenomenon had taken place.

(*b*) The possibility of a precipitin reaction between the guinea-pig serum of the complement and the antilytic serum was eliminated by the use of complement from other sources. Thus, in the following mixtures:

1. Inhibiting serum	0.2 c.c.	Left at 40° C. for 15 minutes.
Human complement	0.2 c.c.	
Salt solution	3.0 c.c.	
2. Inhibiting serum	0.2 c.c.	Left at 40° C. for 15 minutes.
Rabbit complement	0.2 c.c.	
Salt solution	3.0 c.c.	
3. Inhibiting serum	0.2 c.c.	Left at 40° C. for 15 minutes.
Guinea-pig complement..	0.2 c.c.	
Salt solution	3.0 c.c.	

The subsequent addition of sensitized corpuscles resulted in complete inhibition, while control tubes with similar varieties and quantities of complement showed hemolysis. There was one exception to this, which we did not follow in detail. One antilytic serum did not seem to oppose the action of dog complement. We mention this, in passing, without trying to explain it.

It seems reasonable, from the foregoing facts, then, to conclude that the inhibiting action of our serum was due to direct anticomplementary action, using the term merely as differentiating this action from the indirect modes of eliminating complement considered above.

CHEMICAL NATURE OF THE THERMOLABILE ANTICOMPLEMENTARY BODY.

Our first attempt toward the identification of this anticomplementary body with any of the serum components, was based upon Noguchi's success in identifying the thermostable anticomplementary body with the serum lipoids. In our tests, serum diluted ten times with salt solution was shaken for fifteen minutes with ether. As the ether did not readily separate from such a mixture, centrifugalization was now employed, and the supernatant ether was carefully pipetted away. Reëxtraction with ether in the same way was twice repeated. The serum residue was now examined for inhibiting powers, the quantities employed being similar to those which inhibited completely in the original serum. Such experiments showed that absolutely no loss of anticomplementary action had been sustained by ether extraction. It seemed unlikely, therefore, that the serum lipoids, at least those extractable with ether by this rather crude method, should account for the anticomplementary action of our serum.

In order to ascertain whether any of the dialyzable components of the serum were responsible for the anticomplementary action, ten cubic centimeters of diluted anticomplementary serum were dialyzed through parchment for forty-eight hours against frequently changed distilled water. Again, the serum residue which became turbid, probably by the precipitation of globulin in this process, showed absolutely no loss of its antilytic power.

It remained to examine the serum proteids. Five cubic centimeters of the serum were diluted with forty-five cubic centimeters of salt solution, and an equal bulk (fifty cubic centimeters) of a saturated ammonium sulphate solution was added. The precipitated globulins were removed by repeated filtration through a triple thickness of fine filter paper. The filtrate was dialyzed against running water to remove the ammonium sulphate. The substances on the filter paper were scraped off, redissolved in an 0.85 per cent. solution of sodium chloride, and reprecipitated with ammonium sulphate. Finally, they were dried and rapidly rinsed with cold water to remove gross particles of ammonium sulphate. Allowing for losses during this process, the bulk of the globulin precipitate was taken up in thirty cubic centimeters of an 0.85 per cent. salt solution. This solution was tested in varying quantities roughly representing from 0.1 to 0.2 cubic centimeter of the original serum. Complete inhibition of hemolysis resulted. The filtrate after forty-eight hours of dialysis still contained traces of ammonium sulphate, but possessed absolutely no anticomplementary action. These tests were controlled with ammonium sulphate solutions in order to preclude the possibility of hemolysis due to the ammonium sulphate alone, and with ammonium sulphate solutions plus the original antilytic serum, to preclude the possibility of interference with the inhibiting principle on the part of the ammonium sulphate.

It appeared from these experiments that the antilytic principle had been removed from the serum with the globulins.

It now seemed desirable to ascertain whether the antilytic factor of the globulins obtained from the serum was thermolabile, as was the antilytic principle in the original serum.

To determine this point, 1 cubic centimeter of the globulin solution (representing approximately 0.1 cubic centimeter of the original serum) was placed in each of four test tubes, and in each of four others was placed 1 cubic centimeter of the original antilytic serum, after this had been diluted ten times with salt solution. Each of the tubes containing globulin was paired with one containing the antilytic serum, and the pairs were subjected to heat for twenty minutes, one to 50° C., another to 56° C., another to 60° C., and another to 70° C. To each tube was then added 0.1 cubic centimeter of fresh guinea pig complement and 2 cubic centimeters of salt solution. The mixtures were allowed to stand at 40° C. for thirty minutes and sensitized corpuscles were then added.

The result showed that there was a complete parallelism between the globulin solution and the original antilytic serum, neither being affected by 50° C., but the antilytic action of both being removed by temperatures between 56° C. and 60° C. It thus appeared that the thermolabile substance could be removed from the antilytic serum with the globulins, and that the globulins, precipitated from such serum and redissolved, displayed anticomplementary and heat-sensitive properties exactly like those of the original serum.

CONCLUSIONS.

The work recorded above has served to corroborate the observations of other writers, notably of Noguchi, that in many human sera, if not in all, there develop, on standing, anticomplementary bodies which are delicately thermolabile, being inactivated by heating to 56° C. for fifteen to twenty minutes. It has appeared that these bodies may occasionally be present in sera which, after heating, may develop the thermostable anticomplementary body referred to by other authors. It has become evident also that the speed of development of the thermolabile anticomplementary body in different sera is subject to much variation. The thermolabile body appears as the complement disappears. The question arises whether the thermolabile anticomplementary body may not be originally present in the serum, temporarily masked by the native complement. This would seem improbable from the fact that titration has shown that 0.1 and 0.2 cubic centimeter of the inhibiting serum will often inhibit as much as 0.6 cubic centimeter of fresh guinea-pig complement, a quantity superior to the amount of complement present originally in the antihemolytic serum. It seems, therefore, that the anticomplementary body must actually be formed in the serum during the period of preservation.

The thermolabile antihemolytic body studied by us is like the thermostabile body investigated by Noguchi, in that it inhibits alien as well as homologous complement; it is unlike the thermostabile body, however, in that it cannot be absorbed from serum by digestion with red cells, nor does it render the treated cells more resistant to hemolysis.

The thermolabile body may be removed from inhibiting sera by

precipitating the globulins. A solution of the globulins then manifests a thermolabile anticomplementary action.

No relationship between a clinical condition and the appearance of these bodies in the sera, has been found.

As a practical result these studies have shown, as have those of Noguchi, that the complement fixation tests should never be made with certain sera which have been preserved for some time without inactivation.

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BIOCHEMICAL STUDIES ON SO-CALLED SYPHILIS ANTIGEN.[1]

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INTRODUCTION.
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Iodine value and acidity.
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SUMMARY.

INTRODUCTION.

The mechanism of the Wassermann reaction is still unsolved. We know merely this: that complement in the presence of syphilitic antigen may be rendered inactive by one or more substances in the body fluids of a syphilitic or parasyphilitic patient. Our present paper deals with the nature of these antigenic substances.

Wassermann, Neisser, and Bruck discovered that the aqueous extract of the liver of a congenitally syphilitic fetus possessed antigenic properties. Ladislaus Detre found independently, and almost si-

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multaneously, a similar property in the extracts prepared from condyloma and other syphilitic tissues. Subsequent investigations of Marie and Levaditi brought out the fact that an aqueous extract of a non-syphilitic liver has a weaker but similar action.

A remarkable revelation soon followed through the studies of Landsteiner, Müller, and Pötzl, and later through those of Meier and Porges, who discovered that the antigenic substances can be extracted by means of alcohol from the human or animal liver or heart. This finding has opened up an entirely new phase of the doctrine of the well known phenomenon of complement fixation, and separates the Wassermann reaction from the classical examples of the Bordet-Gengou reaction due to specific antigens and antibodies.

Additional studies by Levaditi and Yamanouchi, Meier and Bauer, Fleischmann, Sachs and Rondoni, Noguchi, Browning and McKenzie, Leathes and Fitzgerald, and many others, confirmed and extended the original observations of Landsteiner, Müller, and Pötzl, and of Porges and Meier. As the result of these researches, the following facts may be considered as established: the Wassermann reaction can be produced with lecithin, certain phosphatids of liver, heart, or kidney, and, in a lesser degree, with certain bile salts, all of which substances are extractable with alcohol. It is also contended that the entire alcoholic extract of organs, lecithins, and some phosphatids, do not give as high a percentage of positive reactions in syphilis or metasyphilis as a suitable aqueous extract of syphilitic fetal liver. This seems to indicate that the aqueous extract contains something in addition to the lipoids and salts which makes the reaction stronger and more specific. This assumption, however, has not been proved.

It is well known that some specimens of congenitally syphilitic fetal livers do not manifest antigenic properties. One is compelled, therefore, to make a thorough trial before concluding that an extract may be used as antigen.

In the case of aqueous extracts, the selection of a suitable one requires much care. The same care has not been exercised in selecting alcoholic extracts and lipoids. In the case of the lipoids, especially lecithin, this lack of precaution is due largely to the use of a

definite chemical term. When speaking of lecithin, one is accustomed to think of it as a chemical and to overlook its biological properties. The same is true of phosphatids. This chemical interpretation of the terms has led many investigators to rely on any preparation of lecithin or phosphatids of tissues without biological discrimination. A worker often uses lecithin because it has been recommended by some investigators. Noguchi, who has been criticised for recommending lecithin, has always coupled the term with "selected." For this and other reasons he no longer employs the term lecithin in designating the aceton-insoluble fraction of tissue lipoids.

In testing lipoids derived from beef kidney and fractionated in twenty-one different ways by Professor Edward K. Dunham, one of us (Noguchi) found that only nine possessed antigenic properties. Of the nine, three were soluble in aceton and six (phosphatids) were insoluble. Only one of the latter was composed of lecithin. Noguchi found, moreover, on comparing the aceton-insoluble fraction of the blood clot and serum of syphilitic and normal persons with the corresponding fractions of syphilitic and normal livers, that the antigenic activity of the former was not inferior to that of the latter. Moreover, the aceton-soluble fraction of the liver and especially that of the blood coagulum and serum possessed antigenic properties. After studying its hemolytic and anti-complementary properties, Noguchi advised the exclusive use of the aceton-insoluble fraction instead of the entire unfractionated alcoholic extract, for the reason that the aceton-insoluble lipoids possess the highest antigenic action and are unaccompanied by other undesirable properties. By eliminating the other constituents (such as neutral fats, fatty acids, soaps, and certain proteid materials) contained originally in the alcoholic extract, Noguchi succeeded in avoiding the non-specific complement fixation which occasionally occurred in unheated human serum. Here arose the important question: What is the criterion by which one can judge the antigenic fitness of the aceton-insoluble fraction? Noguchi had met with a number of preparations which were unsuitable either on account of the total absence or weakness of the antigenic properties or on account of the presence in the emulsion of some hemolytic or anti-

complementary substance. These irregularities made it necessary to study analytically a large number of tissues with special reference to the composition of the alcoholic extract—the antigenic, hemolytic, and anticomplementary properties of different fractions, and the chemical constitution of the more important ones.

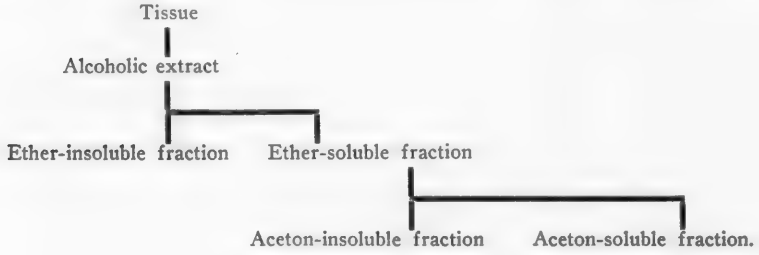
These were the problems which confronted us and they have led to the work which forms the basis of the present article. We believed that it was a matter of great importance to approach the question experimentally, for reducing the problem to a biochemical basis removes from the Wassermann reaction its chief difficulty.

METHOD OF STUDY.

EXTRACTION AND FRACTIONATION.

The organ was cut into very small pieces, mixed with four volumes of 95 per cent. alcohol and preserved in a sealed jar. With occasional shakings, it was left at room temperature for six to seven weeks. The alcohol was then passed through filter paper and a second extract was made by treating the residue with four volumes of absolute alcohol at 37° C. for four days, with daily shaking. The second extract was collected by filtration, united with the first and the whole was placed in a very wide crystallization dish and evaporated by means of a current of air at 37° C. After desiccation the sticky mass was weighed and then treated repeatedly with ether to separate the ether-soluble substances. The ether-insoluble part was weighed and the ether-soluble portion was evaporated and weighed and then dissolved in a small quantity of ether and mixed with ten volumes of water-free aceton. The large amount of precipitate formed was usually separated by careful decantation—sometimes, however, filtration through hardened filter paper was necessary. The aceton-insoluble precipitate (phosphatids) was collected, freed from aceton by evaporation, and weighed. The aceton-soluble fraction (neutral fats, fatty acids, cholesterin, etc.) was freed from aceton by evaporation and weighed.

The various fractions derived from the tissue may be represented schematically as follows:



The method indicated above was considered sufficiently accurate for our purpose, as we intended to resort to a more thorough analysis in our next undertaking.

The solubility in hot alcohol of the ether-insoluble fraction was ascertained for a few specimens. The fractions soluble and insoluble in hot alcohol proved to be soluble in water. The insoluble fraction is composed of certain protein substances which, together with various salts, are held in apparent solution in the alcoholic extract. The hot alcohol-soluble fraction is composed chiefly of salts of saturated and unsaturated fatty acids and of bile acid salts.

The substances contained in the fractions soluble and insoluble in hot alcohol are important sources of error in complement fixation tests.

IODINE VALUE AND ACIDITY.

The iodine value of the aceton-insoluble fraction of each undehydrated specimen was determined by the method of Hübl. For this purpose the light brownish mass, which had been under a layer of water-free aceton in a sealed tube, was taken out of aceton and air-dried until the aceton evaporated. This required about fifteen minutes.

The acidity of the aceton-soluble fraction was determined after the aceton had been completely evaporated. The oily substance was weighed out and 0.025 gm. was dissolved in 10 c.c. of ethyl alcohol and the amount of N/100 potassium hydroxide necessary to neutralize 1 c.c. was determined, the indicator, phenolphthalein, remaining red for one minute.

BIOLOGICAL PROPERTIES.

Systematic and parallel examinations were made to determine the inherent hemolytic, the anticomplementary, and the antigenic properties of the fractionated fats and lipoids.

Preparation of Emulsions.—The emulsions were uniformly 0.25 per cent., that is, 0.025 gm. of material was suspended in 10 c.c. of a 0.9 per cent. sodium chloride solution. In the case of the aceton-soluble fraction, however, accurate work was impossible, for, in isotonic salt solution (0.9 per cent. sodium chloride), it proved exceedingly difficult to make a stable emulsion of this fraction. The aceton-insoluble fraction, however, easily emulsified by mixing salt solution with a concentrated solution of the material in ether or alcohol. Even when the material was not completely soluble in ether or alcohol, a good emulsion was obtained by adding salt solution. The emulsions of the latter were clear and opalescent. They were usually neutral to litmus and remained stable during many months.

Ether-insoluble Fraction.—The quantity of ether insoluble material obtained from different livers varied greatly. Autolyzed organs yielded the largest amount.

The biological factors in the ether insoluble fraction were not studied systematically in the experiments here reported. One of us (Noguchi), however, had already made a special examination of it and found that the hot alcohol-*insoluble* material of this fraction sometimes caused, with a certain human serum, a non-specific fixation of complement, and that the hot alcohol-*soluble* fraction

always exhibited a powerful hemolytic property. The amount of the hot alcohol-soluble fraction in the ether-insoluble material varied greatly, being sometimes very small, sometimes large.

Determination of the Biological Properties.—(*a*) To determine the ***inherent hemolytic property,*** gradually increasing fractions of 1 c.c. of the emulsions were put into a series of test tubes and 0.9 per cent. sodium chloride solution was added to bring the contents of each tube up to 1 c.c. Into each tube was then introduced 0.1 c.c. of a 10 per cent. suspension of washed human corpuscles. The tubes were shaken and incubated at 37° C. for two hours. The quantities of emulsion in the table are for complete hemolysis.

(*b*) To determine the ***anticomplementary property,*** increasing fractions of 1 c.c. of the emulsions, as above, were placed in a series of tubes, and then into each, one unit of complement (fresh guinea pig serum), usually 0.1 c.c. of a 20 per cent. dilution, was introduced and enough salt solution was added to make the contents of each tube equal to 1 c.c. The tubes were incubated at 37° C. for one hour in an air thermostat, or for thirty minutes in a constant temperature water bath. After incubation, to each tube 0.1 c.c. of a 10 per cent. suspension of washed human corpuscles and one unit of antihuman amboceptor (rabbit) were added. The mixture, with frequent shakings, was now incubated for two hours, and the result was recorded. The quantities of emulsion given in the table are for complete inhibition.

(*c*) To determine the ***antigenic property,*** a mixture of strongly positive syphilitic sera from six or more persons was employed. These sera were usually active, but sometimes they were inactivated (*i. e.,* when anticomplementary). It was necessary, first, to determine the smallest amount of serum capable of causing complete fixation, that is, the unit of syphilitic antibody. In order to do this, it was necessary to have a standard antigen emulsion. This we had, and its strength and quality had been proved in connection with an innumerable number of positive as well as non-syphilitic sera. In this way we determined the smallest quantity of syphilitic serum (antibody) that could be detected by using a good antigen, and this quantity of the serum was used for testing the strength of our different preparations of emulsion. In our tests gradually increasing quantities of the emulsion were measured into a series of test tubes, to which were also added one unit of syphilitic antibody and two units of complement, that is, 0.1 c.c. of a 40 per cent. dilution of fresh guinea pig serum. As usual, the contents of each tube were made equal to 1 c.c. by adding salt solution. The mixture was incubated at 37° C. for one hour, and then 0.1 c.c. of a 10 per cent. suspension of washed human corpuscles and two units of antihuman amboceptor were added to each tube. The second incubation was for two hours. The results were taken a few hours after stopping the incubation.

The quantities of emulsion recorded in the accompanying table are those which caused complete fixation. The largest amount of emulsion employed was 0.4 c.c., and any emulsion which, in this quantity, failed to cause fixation was regarded as possessing no antigenic property.

MATERIAL FOR INVESTIGATIONS.

Animal and human livers and animal hearts and brains were studied. The total number of all tissues investigated was eighty-seven. Animal livers were

obtained fresh in the laboratories of the Rockefeller Institute and placed at once in alcohol (95 per cent.). To our collection Dr. Dochez, of the Institute, kindly added the alcoholic extract of an autolyzed dog liver.

Livers were secured from the following animals: rabbits, 4; guinea pigs, 5; and dogs, 4. In addition to these, brains were obtained from eight guinea pigs and two dogs, and hearts from eight guinea pigs. On account of their smallness the brains and hearts of the guinea pigs were extracted together.

Human livers were secured through the courtesy of Dr. James W. Moore, Central Islip State Hospital, New York; Dr. Charles Rusk, Manhattan State Hospital, New York; Dr. John A. Wiseman, Kings Park State Hospital, New York; Dr. Martha Wollstein, Babies' Hospital, New York City; Dr. Mortimer Warren, Roosevelt Hospital, New York City; and Dr. Benjamin Schwartz, Bellevue Hospital, New York City. For their kind coöperation we wish to express our thanks.

The human livers were obtained from patients who had suffered with the following: dementia paralytica, 16; dementia precox, 8; dementia senilis, 2; manic depressive insanity, 1; senile psychosis, 10; epileptic psychosis, 1; cerebral hemorrhage, 1; pulmonary tuberculosis, 4; tubercular meningitis, 2; tubercular peritonitis, 1; erysipelas, 2; acute peritonitis, 4; lobar pneumonia, 3; bronchopneumonia, 1; acute enteritis, 1; carcinoma ventriculi, 1; cirrhosis of liver, 1; pulmonary thrombosis, 1; insufficiency of mitral and aortic valves, 1; congenital heart malformation, 1; severe anemia, 1; cerebral abscess, 1; uremia, 1; and chronic alcoholism, 1. Livers were also secured from an undiagnosed psychiatric patient, from an imbecile, from a nine months fetus, and from a premature child. The total number of human livers employed in these experiments was seventy.

With one exception all autopsies were performed within twenty-four hours after death. In the exceptional case eighty-two hours had elapsed. The livers were collected at autopsy and put immediately into four volumes of 95 per cent alcchol.

Our complete experimental protocols are given in one general table and the results are described in the following pages. The results are further analyzed according to certain special considerations, such as the frequency and distribution of various biological properties of the fractionated tissue extracts, with reference to clinical diagnosis, pathological conditions of the tissues, and the age of the patients.

RESULTS OF EXPERIMENTS.

I. GENERAL PRESENTATION AND DISCUSSION OF RESULTS.

In Table I we have brought together all experimental data in such form that one can easily see the quantities of the different fractions of our tissues and their biological properties. It also gives the iodine number and acidity of some of the fractions of each specimen.

TABLE I.

		Age.	Fractionation.						Biological properties of the ether-soluble tissue constituents, together with determination of the iodine values and acidity.							
									Aceton-insoluble fraction.				Aceton-soluble fraction.			
									0.25 per cent. emulsion				0.25 per cent. emulsion.			
			Weight of tissue in gm.	Weight of substances extracted with alcohol in gm.	Weight of substances insoluble in ether in gm.	Weight of substances soluble in ether in gm.	Weight of substances insoluble in aceton in gm.	Weight of substances soluble in aceton in gm.	Antigenic (in c.c.).	Hemolytic (in c.c.).	Anticomplementary (in c.c.).	Iodine value.	Antigenic (in c.c.).	Hemolytic (in c.c.).	Anticomplementary (in c.c.).	Acidity N/10 potassium hydroxide.
1	Liver (normal): dementia paralytica, carbon monoxide poisoning 2 months before death	62 yr.	25	2.4	0.5	1.9	0.77	1.1	0.02			59.00		0.03		0.5
2	Liver: dementia paralytica (tabetic), pneumonia, nephritis, syphilis	47 yr.	24	2.7	0.2	1.5	0.92	0.56	0.02			54.61	0.1	0.15		0.25
3	Liver (passive congestion): dementia paralytica	35 yr.	24	2.15	0.4	1.69	0.85	0.32	0.04			46.99	0.1	0.15		0.175
4	Liver (normal): dementia paralytica, broncho-pneumonia, nephritis	62 yr.	23	2.	0.35	1.65	0.85	0.75	0.04			51.43	0.05	0.15		0.25
5	Liver (anemic): dementia paralytica, pachymeningitis hemorrhagica, pneumonia	35 yr.	25	1.69	0.35	1.3	0.62	0.62	0.05			38.78			0.1	0.2
6	Liver (passive congestion): dementia paralytica, pneumonia, pulmonary emphysema, syphilis	40 yr.	23	1.95	0.52	1.29	1.02	0.25	0.03		0.4	44.45	0.1	0.15		0.25
7	Liver (passive congestion): dementia paralytica (tabetic), acute mitral valvulitis, miliary abscesses of kidneys, cystitis	36 yr.	26	2.95	1.6	1.4	0.95	0.35	0.04			46.85	0.05	0.2	0.1	0.275
8	Liver (passive congestion): dementia paralytica, chronic aortitis, double pyelonephritis	48 yr.	28	2.5	0.75	1.75	0.95	0.8	0.04		0.2	49.47	0.05		0.15	0.35

9	Liver (normal): dementia paralytica, acute nicotin poisoning . .	50 yr.	25	1.95	0.45	1.5	0.9	0.6	0.05		0.2	49.47		0.15	0.2	0.175
10	Liver (passive congestion): dementia paralytica, chronic nephritis	57 yr.	28	2.35	0.95	1.4	0.8	0.5	0.04		0.2	44.45	0.15		0.2	0.225
11	Liver (passive congestion): dementia paralytica, pulmonary tuberculosis, nephritis	51 yr.	37	2.05	1.15	0.85	0.45	0.4	0.04		0.15	46.35	0.1	0.2		0.225
12	Liver (passive congestion): dementia paralytica, aortitis syphilitica, broncho-pneumonia	55 yr.	31	1.9	0.69	1.2	0.82	0.36	0.05			43.81	0.1	0.15	0.2	0.25
13	Liver (normal): dementia paralytica, pneumonia, nephritis	58 yr.	28	1.69	0.35	1.31	0.74	0.47	0.05		0.2	38.10	0.1	0.15	0.2	0.225
14	Liver (fatty): dementia paralytica, chronic nephritis	50 yr.	32	2.45	0.91	1.53	0.92	0.5	Slight fixation			31.75		0.1	0.2	0.225
15	Liver (passive congestion): dementia paralytica, chronic nephritis, arteriosclerosis	60 yr.	29	2.1	0.75	1.34	0.6	0.64	0.03			50.80	0.1	0.15	0.2	0.25
16	Liver (fatty): dementia paralytica, aortitis syphilitica, pulmonary edema	49 yr.	30	2.	0.68	1.3	0.61	0.58	Slight fixation			31.11		0.1	0.2	0.3
17	Liver (normal): dementia senilis, chronic nephritis, ascites, hydrothorax	65 yr.	30	3.2	0.3	2.85	1.35	1.47	0.04			43.81		0.1		0.15
18	Liver (normal): dementia senilis (?), brown atrophy of heart, thrombosis of aorta, arteriosclerosis	60 yr.	22	3.33	1.2	2.1	1.2	0	0.05		0.2	40.64	0.1		0.15	0.125
19	Liver (sclerosis and perihepatitis): senile psychosis, pulmonary tuberculosis, nephritis, amyloid degeneration of spleen	59 yr.	23	3.	1.	2.	0.6	1.4	0.02			47.62	0.2			0.2
20	Liver (normal): senile psychosis, generalized arteriosclerosis, broncho-pneumonia, nephritis, gangrene of foot	63 yr.	25	2.3	0.43	1.8	0.97	0.8	0.05			38.73		0.25		0.225
21	Liver (normal): senile psychosis, pulmonary tuberculosis, chronic nephritis	63 yr.	25	2.	0.4	1.55	0.83	0.72	0.03		0.4	47.62	0.2			0.25

TABLE I (CONTINUED).

		Age.	Fractionation.						Biological properties of the ether-soluble tissue constituents, together with determination of the iodine values and acidity.							
									Aceton-insoluble fraction.				Aceton-soluble fraction.			
									0.25 per cent. emulsion				0.25 per cent. emulsion.			
			Weight of tissue in gm.	Weight of substances extracted with alcohol in gm.	Weight of substances insoluble in ether in gm.	Weight of substances soluble in ether in gm.	Weight of substances insoluble in aceton in gm.	Weight of substances soluble in aceton in gm.	Antigenic (in c.c.).	Hemolytic (in c.c.).	Anticomplementary (in c.c.).	Iodine value.	Antigenic (in c.c.).	Hemolytic (in c.c.).	Anticomplementary (in c.c.).	Acidity N/10 potassium hydroxide.
22	Liver (normal): senile psychosis, hypertrophy of heart	63 yr.	25	2.73	1.15	1.55	1.	0.55	0.04			48.89	0.05	0.2	0.1	0.225
23	Liver (passive congestion): senile psychosis, pneumonia hypostatica	64 yr.	24	2.6	0.75	1.87	0.8	1.05	0.05			43.81		0.1		0.25
24	Liver (normal): senile psychosis, moderate cerebral atrophy, general arteriosclerosis, chronic nephritis	88 yr.	25	2.15	0.85	1.25	0.75	0.5	0.05		0.2	44.45		0.2		0.2
25	Liver (normal): senile psychosis, chronic nephritis, choleocystitis	86 yr.	25	2.48	0.4	2.02	1.05	0.95	0.03			49.47	0.3			0.175
26	Liver (82 hours post mortem) senile psychosis, chronic nephritis, cholelithiasis	67 yr.	29	1.95	1.05	0.95	0.42	0.52	0.05			42.54		0.25		0.2
27	Liver (fatty): senile psychosis, dilatation of heart, infarct of kidney	65 yr.	30	1.85	0.57	1.27	0.69	0.58	No fixation			21.94				
28	Liver (passive congestion): senile psychosis	60 yr.	30	2.25	0.32	1.9	0.82	1.05	0.04			44.45	0.1	0.1	0.2	0.3
29	Liver (normal): dementia precox, carcinoma of pancreas	55 yr.	24	3.	1.13	1.82	0.57	1.25	0.04			46.35	0.05	0.1		0.25
30	Liver (normal): dementia precox, miliary tuberculosis of lungs and spleen	44 yr.	24	2.95	0.8	2.07	0.9	1.1	0.03			50.80		0.05		0.225

31	Liver (passive congestion): dementia precox, chronic nephritis	62 yr.	25	2.3	0.8	1.45	0.9	0.5	0.04		0.4	50.03		0.07	0.05	0.175
32	Liver (passive congestion): dementia precox, broncho-pneumonia	50 yr.	21	1.65	0.4	1.3	0.65	0.65	0.04			39.37		0.05		0.225
33	Liver (normal): dementia precox, pulmonary tuberculosis	50 yr.	25	2.5	1.15	1.35	0.9	0.45	0.02			56.57		0.05		0.35
34	Liver (normal): dementia precox	27 yr.	30	3.	1.3	1.65	0.95	0.7	0.04			48.26	0.15		0.2	0.175
35	Liver (normal): dementia precox, pulmonary tuberculosis	31 yr.	27	2.95	1.2	1.75	0.95	0.8	0.02			59.00		0.1		0.225
36	Liver (normal): dementia precox, pulmonary tuberculosis	37 yr.	13	0.77	0.15	0.6	0.27	0.31	0.05			38.19	0.1	0.15	0.2	0.2
37	Liver (very soft and friable) epileptic psychosis; died in status; broncho-pneumonia	52 yr.	30	2.6	0.9	1.65	1.2	0.45	0.02			52.07		0.05		0.25
38	Liver (normal): manic depressive insanity, septicemia, abscesses of kidney	47 yr.	35	1.47	0.3	1.15	0.85	0.3	0.04		0.2	43.81		0.1		0.25
39	Liver (fatty); imbecile, peritonitis septica	45 yr.	30	2.3	0.22	2.2	0.84	1.29	0.07			34.35		0.05	0.2	0.25
40	Liver (marked passive congestion): cerebral hemorrhage, pulmonary tuberculosis	40 yr.	32	3.2	0.97	2.15	0.95	1.19	0.05		0.4	37.46	1.15		0.3	0.2
41	Liver (normal): pulmonary tuberculosis	3 yr.	30	2.3	1.1	1.15	0.4	0.75		0.3			0.05	0.01		0.25
42	Liver (normal): pulmonary tuberculosis	3 yr.	30	2.05	0.7	1.3	0.8	0.5	0.03		0.2	49.47	0.1		0.2	0.15
43	Liver (normal): tubercular meningitis	4 yr.	30	3.2	1.2	2.	1.2	0.75	0.03		0.2	56.54	0.075	0.1		0.25
44	Liver (normal): pulmonary tuberculosis	35 yr.	35	4.2	2.5	1.7	0.6	1.05	0.05			34.47	0.025			0.45
45	Liver (normal): pulmonary tuberculosis	18 yr.	30	2.05	0.75	1.3	0.7	0.6	0.04			41.91	0.1	0.2		0.15
46	Liver (normal): tubercular peritonitis	30 yr.	30	3.	1.35	1.65	0.85	0.8	0.04		0.2	48.73		0.025		0.375
47	Liver (normal): tubercular meningitis	8 mo.	34	1.6	0.4	1.2	0.85	0.32	0.02		0.2	53.34		0.05		0.2
48	Liver (normal): erysipelas	7 mo.	32	1.87	1.1	0.75	0.6	0.15	0.05			42.54		0.025		0.3
49	Liver (normal): erysipelas	6 wk.	32	2.95	0.6	2.2	0.75	1.4	0.02		0.2	50.80		0.05		0.2

TABLE I (CONTINUED).

		Age.	Fractionation.						Biological properties of the ether-soluble tissue constituents together with determination of the iodine values and acidity.							
									Aceton-insoluble fraction.				Aceton-soluble fraction.			
									0.25 per cent. emulsion				0.25 per cent. emulsion.			
			Weight of tissue in gm.	Weight of substances extracted with alcohol in gm.	Weight of substances insoluble in ether in gm.	Weight of substances soluble in ether in gm.	Weight of substances insoluble in aceton in gm.	Weight of substances soluble in aceton in gm.	**Antigenic** (in c.c.).	Hemolytic (in c.c.).	Anticomplementary (in c.c.).	**Iodine value.**	Antigenic (in c.c.).	Hemolytic (in c.c.).	Anticomplementary (in c.c.).	Acidity N/10 potassium hydroxide.
50	Liver (normal): acute peritonitis	3 mo.	25	1.9	0.37	1.5	0.95	0.5	**0.02**			**52.04**	0.05	0.1		0.2
51	Liver (normal): acute peritonitis, gastric ulcer	19 yr.	25	1.8	0.8	1.	0.8	0.2	**0.04**		0.1	**40.00**		0.025		0.425
52	Liver (normal): acute peritonitis, typhoid fever	25 yr.	32	2.65	1.05	1.55	0.9	0.65	**0.03**			**45.72**		0.025		0.275
53	Liver (normal): acute peritonitis	25 yr.	30	2.8	0.8	1.9	0.95	0.92	**0.02**			**50.80**	0.05	0.075		0.275
54	Liver (normal): pneumonia and influenza	7 mo.	30	2.54	0.7	1.8	1.25	0.45	**0.04**			**39.37**	0.05	0.1		0.25
55	Liver (normal): pneumonia	34 yr.	24	2.98	1.	1.95	0.95	1.	**0.03**		0.2	**44.45**	0.2	0.25		0.4
56	Liver (congested): pneumonia	50 yr.	32	2.27	0.85	1.47	0.8	0.65	**0.04**			**41.27**		0.1		0.275
57	Liver (normal): pneumonia	7 mo.	25	2.55	0.8	1.75	0.75	1.05	**0.05**			**40.00**	0.075	0.1		0.2
58	Liver (normal): acute enteritis	3 wk.	28	2.1	0.57	1.73	1.2	0.5	**0.02**		0.2	**53.37**		0.1		0.375
59	Liver (normal): cancer of stomach	40 yr.	30	3.35	1.53	1.8	1.28	0.5	**0.02**		0.2	**54.61**		0.1		0.25
60	Liver (normal): cirrhosis of liver	55 yr.	35	2.95	0.97	2.05	1.1	0.9	**0.02**			**50.80**		0.025		0.225
61	Liver (normal): pulmonary thrombosis	50 yr.	25	1.85	0.65	1.25	0.65	0.58	**0.05**			**39.37**	0.2			0.2
62	Liver (passive congestion): cardiac insufficiency	60 yr.	30	3.73	2.3	1.45	0.75	0.7	**0.05**			**38.10**		0.025		0.375
63	Liver (normal): malformation of heart; serum reaction positive	1 wk.	30	2.3	1.05	1.25	0.95	0.3	**0.03**		0.2	**50.80**	0.15			0.225
64	Liver (normal): grave anemia	30 yr.	30	4.85	3.55	1.3	0.3	1.	**0.02**			**53.34**		0.025		0.5
65	Liver (normal): cerebral abscess	36 yr.	30	3.2	2.78	2.35	1.28	1.07	**0.02**		0.1	**60.27**	0.1	0.15		0.2
66	Liver (normal): uremia	50 yr.	25	2.85	1.05	1.8	1.15	0.65	**0.04**			**41.27**		0.025		0.475
67	Liver (normal): alcoholism	53 yr.	24	3.8	1.3	2.5	0.6	1.85	**0.04**		0.2	**58.36**		0.15	0.1	0.25

68	Liver (normal): premature child, atelectasis		30	3.1	1.1	2.1	0.9	1.1	0.02			54.61	0.05	0.15		0.275
69	Liver (normal): 9 month fetus; mother, negative serum reaction		30	2.8	0.8	1.9	0.65	1.17	0.02			55.88		0.1		0.25
70	Liver (normal): died in excitement; hemorrhagic infarct of the lungs	35 yr.	26	2.55	0.42	2.08	1.1	0.9	0.02			64.73		0.025		0.2
71	Liver (rabbit)	Full grown	32	2.1	0.7	1.45	0.95	0.5	0.04		0.2	44.45	0.15	0.15	0.2	0.225
72	Liver (rabbit)	"	30	2.75	0.9	1.85	0.75	1.1	0.04		0.15	41.27	0.15	0.2	0.2	0.25
73	Liver (rabbit)	"	34	2.8	0.6	2.2	1.5	0.7	0.03		0.15	47.63		0.15	0.1	0.225
74	Liver (rabbit)	"	35	3.05	1.15	1.9	1.35	0.52	0.05		0.2	34.47			0.2	0.2
75	Liver (guinea pig)	"	30	2.3	0.7	1.58	0.87	0.7	0.04			44.45	0.15		0.2	0.2
76	Liver (guinea pig)	"	30	2.2	0.5	1.7	1.2	0.5	0.04		0.1	41.90	0.15		0.25	0.225
77	Liver (guinea pig)	"	25	1.2	0.5	0.7	0.5	0.2	0.04		0.2	39.37		0.1		0.3
78	Liver (guinea pig)	"	32	2.38	0.9	1.4	1.1	0.3	0.04			42.91			0.2	0.2
79	Liver (guinea pig)	"	25	2.1	0.7	1.45	1.05	0.4	0.04		0.15	43.18		0.2	0.2	0.175
80	Liver (dog)	"	30	1.85	0.7	1.15	0.7	0.45	0.03		0.2	46.35	0.15	0.15		0.225
81	Liver (dog)	"	30	2.42	1.1	1.32	0.92	0.45	0.05			38.10	0.15		0.2	0.225
82	Liver (dog)	"	30	2.1	0.8	1.3	0.7	0.6	0.03		0.2	46.35	0.05	0.1		0.25
83	Liver (dog): autolyzed			7.	4.85	2.1	1.25	0.8	0.02			69.85		0.03	0.15	0.5
84	Heart (from 8 guinea pigs)	"	8	0.98	0.23	0.75	0.53	0.22	0.03		0.2	49.47	0.15		0.2	0.175
85	Brain (from 8 guinea pigs)	"	20	2.52	0.55	1.95	1.3	0.65	0.1		0.2	57.78	0.15		0.2	0.2
86	Brain (dog)	"	50	5.3	1.5	3.8	2.4	1.4	0.07		0.15	64.73	0.1	0.25	0.2	0.175
87	Brain (dog)	"	50	4.8	1.25	3.5	2.2	1.3	0.07		0.15	62.23			0.2	0.15

Summarizing the results recorded in Table I, the following figures for the alcohol-extractable constituents are obtained:

2,081 grams of liver (70 different specimens from man) yielded:

(*a*) 175.50 grams of substances, equal to 8.4 per cent., which was divided into
(*b*) 60.48 grams of ether-insoluble substances, equal to 2.9 per cent., and
(*c*) 112.87 grams of ether-soluble substances, equal to 5.4 per cent., of which
(*d*) 58.91 grams were aceton-insoluble, equal to 2.8 per cent., and
(*e*) 61.24 grams were aceton-soluble, equal to 2.9 per cent.

363 grams of animal liver (four rabbits, five guinea pigs, three dogs) yielded:

(*a*) 26.65 grams of substances, equal to 7.3 per cent., which was divided into
(*b*) 9.25 grams of ether-insoluble substances, equal to 2.5 per cent., and
(*c*) 17.40 grams of ether-soluble substances, equal to 4.8 per cent., of which
(*d*) 11.59 grams were aceton-insoluble, equal to 3.2 per cent., and
(*e*) 6.02 grams were aceton-soluble, equal to 1.6 per cent.

Fluctuations in the amounts of the entire extracts and also in those of different fractions were considerable in different specimens of human livers. The maximum and minimum were as follows:

	Maximum.	Minimum.
Entire extract before fractionation	16.0 per cent. (No. 64)	4.0 per cent. (No. 38)
Ether-insoluble substances	10.0 per cent. (No. 64)	0.8 per cent. (No. 2)
Aceton-insoluble substances	4.5 per cent. (No. 17)	1.0 per cent. (No. 64)
Aceton-soluble substances	8.0 per cent. (No. 67)	0.8 per cent. (No. 51)

The extracts from animal livers showed the following relations:

	Maximum.	Minimum.
Entire extract before fractionation.	9.0 per cent. (No. 74)	4.8 per cent. (No. 77)
Ether-insoluble substances	3.0 per cent. (No. 74)	1.7 per cent. (No. 73)
Aceton-insoluble substances	4.0 per cent. (No. 79)	2.0 per cent. (No. 77)
Aceton-soluble substances	3.0 per cent. (No. 72)	0.8 per cent. (No. 77)

The weight of substances extractable by alcohol from different livers varies greatly, the variations being greater in extracts from human than in those from animal livers. This striking difference was probably due to the fact that the human specimens were pathological and had undergone a more pronounced alteration after death, the main one, no doubt, being a rapid autolysis.

The ether-insoluble substances from human livers showed the widest range of variation, the minimum per cent. being less than one-twelfth of the maximum. In the case of animals the minimum per cent. of the ether-insoluble substance varied from the maximum by less than one-half.

The variation in the amounts of the aceton-insoluble fraction was not so marked as in that of the ether-insoluble fraction, the maximum per cent. being

only four and a half times the minimum. The liver from a patient who had had severe anemia yielded the smallest aceton-insoluble fraction. The variation in the aceton-insoluble fraction of animal livers was small. Finally, the aceton-soluble fraction was seen to vary greatly, especially in human livers, where the maximum was ten times the minimum.

This great variation, according to Leathes, who studied the metabolism of fats with special reference to the liver in man and animals, was to be expected. It is produced through the transportation of stored fats (adipose) to the liver, in various pathological conditions, in order to convert the inert fats into active fats by desaturation of double carbon bonds. Not pathological conditions only, but physiological ones also, are capable of producing considerable fluctuations in this fraction.

It is noteworthy that the ether-insoluble fraction in autolysed liver of dog 83 is enormously large in comparison with the aceton-insoluble and aceton-soluble fraction which it yielded.

The aceton-insoluble fraction obtained from the hearts of guinea pigs is seen to be quite large, amounting to 6 per cent., while the aceton-soluble fraction is only 2.8 per cent., although in this percentage is included a certain amount of connective tissue fats.

The brains of guinea pigs and dogs yielded about 5 to 6 per cent. of aceton-insoluble fractions and 2 to 3 per cent. of aceton-soluble fractions, while in dogs and guinea pigs the ether-insoluble fraction in the brain extract reached 2 to 3 per cent.

The point of chief interest is the consideration of the extracts from the point of view of their biological behavior. In Table I the quantities of the aceton-insoluble and aceton-soluble fractions of each extract are recorded for hemolytic, anticomplementary, and antigenic activity. The blank spaces in the three columns signify that there was no action when a maximum dose of 0.4 cubic centimeter of each emulsion was used.

Aceton-soluble Fraction.—The majority of the emulsions of the aceton-soluble fraction were hemolytic. Of seventy specimens fifty-four (nearly 70 per cent.) showed this activity. Emulsion No. 41 was the strongest, 0.01 cubic centimeter causing complete hemolysis. In the case of the weakest emulsion, 0.25 cubic centimeter was required to bring about complete hemolysis.

The hemolytic activity did not, as one might suppose, vary with the acidity of the emulsion. In spite of being acid, certain specimens exerted no hemolytic activity. The nature of the acid present is very important, for Noguchi showed some years ago that the hemolytic activity of unsaturated fatty acids is nearly ten times stronger than the normal or saturated fatty acids. It is probable,

therefore, that the emulsions that here proved so strongly hemolytic contained a relatively large quantity of the unsaturated fatty acids. This point, however, requires further study.

The biological evidence for attributing hemolytic activity in some instances to the presence of large quantities of unsaturated fatty acids is, to a certain extent, supported by the results of Leathes, who has shown, by testing the iodine value of various liver extracts, that the quantities of saturated and unsaturated fatty acids in different specimens vary greatly.

In the aceton-soluble fraction, twenty extracts (28 per cent.) showed anticomplementary properties. This activity was manifested by quantities that varied from 0.03 to 0.5 cubic centimeter, the usual quantities being 0.1 to 0.2 cubic centimeter.

Antigenic Properties.—Of seventy specimens thirty-four (about 50 per cent.) manifested antigenic properties. In five specimens the antigenic activity was the only one observed. In the remaining ones, however, it was associated with other properties; in six, with an anticomplementary action; in sixteen, with a hemolytic property; and in seven with both hemolytic and anticomplementary properties.

It should be understood that those emulsions which possessed either hemolytic or anticomplementary properties, or both, were examined for their antigenic property in the quantities which were too small to manifest a hemolytic or anticomplementary action. The emulsions which were antigenic without the other two properties were generally weak; that is, they were active when employed in doses of 0.15, 0.2 and 0.3 cubic centimeter, and were very weak when compared with the aceton-insoluble fractions which will be mentioned below.

The results obtained with the aceton-soluble fraction of *animal livers* are similar to those obtained with the human livers, except that the presence of the anticomplementary property was more frequent in the animal specimens. In examining thirteen livers the anticomplementary property was found in ten (77 per cent.), the hemolytic property in eight (66 per cent.), and the antigenic property in seven (53 per cent.). Every emulsion showed either the hemolytic or the anticomplementary property, or both. Every specimen was acid.

The aceton-soluble fractions of the hearts and brains of guinea-pigs and dogs were in all respects comparable to those of the animal livers just described.

Aceton-insoluble Fraction.—In considering the results obtained with the aceton-insoluble fraction, one sees that the column for the records of the hemolytic property is, with one exception (No. 41) quite blank. In the exceptional case the figures 0.3 cubic centimeter are recorded. This means that sixty-nine out of seventy different emulsions had no hemolytic action when used in the large dose of 0.4 cubic centimeter. Emulsion No. 41 was hemolytic in the dose of 0.3 cubic centimeter. In the corresponding column of the seventy aceton-soluble fractions, fifty-four (70 per cent.) show hemolysis. The absence of the hemolytic activity is characteristic of the aceton-insoluble fraction, while the presence of this activity is characteristic of the aceton-soluble fraction. In the seventy specimens examined, the anticomplementary property was present in twenty-four (34 per cent.). It was, therefore, somewhat more frequently present here than in the aceton-soluble fraction. The anticomplementary property may be present or absent in the aceton-soluble or in the aceton-insoluble fractions derived from the same liver. Its presence in one fraction is, however, quite independent of its presence in the other.

Concerning the aceton-insoluble fractions, the following may be stated: the antigenic property was present in sixty-six liver fractions (94 per cent.). It was absent or extremely weak in three fatty livers, and in the fourth, from a case of tuberculosis, the extract could not be used on account of its hemolytic activity.

Of the sixty-six preparations that possessed an antigenic property, twenty-four (34 per cent.) had an auxiliary anticomplementary action, while forty-two (60 per cent.) were entirely devoid of this.

2. THE RELATION BETWEEN THE ANTIGENIC AND ANTICOMPLEMENTARY PROPERTIES OF THE ACETON-INSOLUBLE FRACTIONS.

Table II shows the anticomplementary property of twenty-four emulsions. These emulsions can not be recommended for the quantitative estimation of complement fixation, although some of them could be used for routine diagnosis for the reason that the amount of the extract necessary to exhibit the antigenic property, differed sufficiently from that which would mani-

TABLE II.

Antigenic titers.	Anticomplementary titers.	Number of preparations of each group.	Ratio of the antigenic to the anticomplementary titers.	Remarks.
0.02 c.c.	0.1 c.c.	1	1: 5	Unsuitable for quantitative work.
	0.2 c.c.	4	1:10	Unsuitable for quantitative work.
0.03 c.c.	0.2 c.c.	4	1: 6.6	Unsuitable for quantitative work.
	0.4 c.c.	3	1:13.3	Unsuitable for quantitative work.
0.04 c.c.	0.1 c.c.	1	1: 2.5	Unsuitable.
	0.15 c.c.	1	1: 3.75	Unsuitable.
	0.2 c.c.	5	1: 5	Unsuitable for quantitative work.
	0.4 c.c.	1	1:10	Unsuitable for quantitative work.
0.5 c.c.	0.2 c.c.	4	1:4	Unsuitable.
	0.4 c.c.	1	1:8	Unsuitable for quantitative work.
		24		Unsuitable 6 Conditional 18 24

fest a disturbing anticomplementary action. The fact that in the fixation test *two units* of complement and of amboceptor are prescribed, renders negligible the possibility of an interference by the anticomplementary property. The anticomplementary effect no longer disturbs when the quantity corresponding to one-third of its titer is tested against *one unit* of complement. Where the relation of the antigenic titer to the anticomplementary titer is as close as 1:2.5, 1:3.75, or 1:4, the emulsion should not be employed even for routine work. It may be mentioned that Wassermann and his pupils determined the minimum quantity of liver extract in which the anticomplementary action could be detected, and they recommended the use of half of this quantity in carrying out the Wassermann reaction.

This principle, while safeguarding the reaction from a non-specific inhibition due to the anticomplementary property, leaves the standard of the antigenic strength of the extract undefined. The quantity they use may contain one antigenic unit, or it may contain several. If the antigenic unit is determined with a strongly positive serum (e. g., six antibody units), this unit is no longer able to detect weaker reactions (e. g., one or two antibody units), while it is easy to do so if the antigenic unit is adjusted to weaker sera.

The results obtained with animal livers agree, in general, with those obtained with human livers. None of the emulsions were hemolytic. On the other hand, the anticomplementary property was present more frequently. All except one emulsion (No. 76) could be used for routine diagnostic work. The antigenic titers were somewhat inferior to those found with the human specimens. The autolyzed liver of dog 83, although having a high antigenic titer, yielded an emulsion free from hemolytic or anticomplementary

properties. The emulsion prepared from guinea-pig hearts possessed a weak anticomplementary and a strong antigenic property.

The biological characteristics of the aceton-insoluble fraction of dog and guinea-pig brains were quite different from those of the liver extracts. While devoid of a hemolytic property, these emulsions were anticomplementary and possessed a slight antigenic property. They are, therefore, unfit for use in the complement fixation test.

The estimation of the iodine absorption in these preparations seems to indicate that there is a certain relationship between the iodine value and the antigenic activity. With few exceptions all preparations with a high antigenic value possessed a high iodine value, and the preparations without antigenic value gave the lowest iodine value. The brain preparations, which usually had a higher iodine value, did not conform to the above generalization, as they had only feeble antigenic values. At present, no explanation for this difference can be given. It is, however, not improbable that the unsaturated carbon bonds in the brain lipoids are situated in different positions from those in the antigenically active lipoids of the liver or heart. Further experimental work, however, is required to determine this point.

3. THE ASSOCIATION BETWEEN CLINICAL CONDITIONS AND THE ANTIGENIC AND IODINE VALUES OF THE ACETON-INSOLUBLE FRACTIONS.

In Table III the clinical diagnosis of the patients from whom the livers were obtained is shown together with an estimation of the comparative frequency and distribution of the antigenic and anticomplementary properties in the aceton-insoluble fractions of these livers. At the bottom of the table we have recorded the figures showing the average iodine values for different antigenic titers and also the maximum and the minimum for each antigenic titer.

4. THE ASSOCIATION OF PATHOLOGICAL CONDITIONS OF THE LIVER WITH THE ANTIGENIC VALUE OF THE ACETON-INSOLUBLE FRACTIONS.

An examination of Table IV does not enable us to discover a definite relation between the antigenic value and the pathological conditions of the livers, except that the fatty livers in no instance yielded a serviceable extract. It is probable that the fatty acid radicals, constituting the lipoids of a fatty liver, belong chiefly to the saturated varieties. The low iodine values of these lipoids are in favor of this hypothesis.

TABLE III.

Mode of Distribution of Different Preparations (Aceton-insoluble Fraction) Arranged according to the Clinical Diagnosis.

	Number of preparations.	Antigenic titer of 0.02 c.c.		Antigenic titer of 0.03 c.c.		Antigenic titer of 0.04 c.c.		Antigenic titer of 0.05 c.c.		Antigenic titer of 0.07 c.c.		Antigenic titer of 0.1 c.c.		Preparations without antigenic value.
		Not anti-complementary.	Anticomplementary.	Not anti-complementary.	Anticomplementary.	Not anti-complementary.	Anticomplementary.	Not anti-complementary.	Anticomplementary.	Not anti-complementary.	Anticomplementary.	Not anti-complementary.	Anticomplementary.	
General paralysis	16	2		1	1	3	3	2	2					2
Senile dementia	2								1					
Senile psychosis	10	1		1	1	2		3	1					1
Dementia precox	8	1		1		3	1	1						
Epileptic psychosis	1	1												
Manic depressive insanity	1						1							
Imbecility	1									1				
Cerebral hemorrhage	1								1					
Pulmonary tuberculosis	4				1	1		1						1
Tubercular meningitis	2		1		1									
Tubercular peritonitis	1		1											
Erysipelas	2			1			1	1						
Acute peritonitis	4	2			1	2								
Pneumonia	4							1						
Acute enteritis	1		1											
Gastric carcinoma	1		1											
Chronic alcoholism	1						1							
Cirrhosis of liver	1	1												
Pulmonary thrombosis	1							1						
Cardiac insufficiency	1							1						
Malformation of heart	1				1									
Grave anemia	1	1												
Cerebral abscess	1		1											
Uremia	1					1								
Premature child?	1	1												
Fetus ?	1	1												
Pulmonary infarct	1	1												

	Number of preparations.	Antigenic titer of 0.02 c.c.		Antigenic titer of 0.03 c.c.		Antigenic titer of 0.04 c.c.		Antigenic titer of 0.05 c.c.		Antigenic titer of 0.07 c.c.		Antigenic titer of 0.1 c.c.		Preparations without antigenic value.
		Not anti-complemen-tary.	Anticom-plementary.	Not anti-complemen-tary.	Anticom-plementary.	Not anti-complemen-tary.	Anticom-plementary.	Not anti-complemen-tary.	Anticom-plementary.	Not anti-complemen-tary.	Anticom-plementary.	Not anti-complemen-tary.	Anticom-plementary.	
Rabbit liver	4				1		2		1					
Guinea pig liver	5					2	3							
Dog liver	3				2			1						
Dog liver (autolyzed)	1	1												
Total	83	14	5	4	9	14	12	12	6	1				4
Anticomplementary in doses of														
0.1 c.c.			1				2							
0.15 c.c.					1		3							
0.2 c.c.			4		6		7		5					
0.4 c.c.					2		1		1					
Average iodine value		55.43		48.50		44.50		40.17		34.35				28.26
Highest iodine value		69.85		56.54		58.36		49.47						31.75
Lowest iodine value		47.62		44.45		40.00		34.47						21.94

TABLE IV.

Liver.	Frequency and distribution of different preparations for the biological properties.											Distribution of biological properties among the pathological conditions.			
	Antigenic titers of the preparations.														
	0.02 c.c.		0.03 c.c.		0.04 c.c.		0.05 c.c.		0.07 c.c.						
	Not anticomplementary.	Anticomplementary.	Not anticomplementary.	Anticomplementary.	Not anticomplementary.	Anticomplementary.	Not anticomplementary.	Anticomplementary.	Not anticomplementary.	Anticomplementary.	Preparations without antigenic value in dose of 0.1 c.c.	Preparations without anticomplementary action.	Preparations with anticomplementary action.	Preparations without antigenic value.	Total.
Human.															
Normal	9	5	3	5	8	4	6	4			1	26	18	1	45
Passive congestion			1	1	5	4	3	1				9	6		15
Anemic	1						1					2			2
Soft and friable	1											1			1
Cirrhotic	2											2			2
Fatty									1		3	1			4
82 hr. after death							1					1		3	1
Guinea pig, rabbit, dog.															
Anemic (bled)				2									2		2
Autolyzed	1											2			2
Anemic (bled)				1		2		1					4		4
Anemic (bled)					2	3						2	3		5
Total	14	5	4	9	15	13	11	6	1		4	46	33	4	83
	83														

TABLE V.

Frequency and Distribution of Different Preparations (Aceton-insoluble Fraction) Arranged according to Age of Patients.

	Distribution of different preparations with reference to biological properties.											Frequency of biological activity for different ages.			
	Preparations with the antigenic titers of														
	0.02 c.c.		0.03 c.c.		0.04 c.c.		0.05 c.c.		0.07 c.c.						
	Not anticomplementary.	Anticomplementary.	Not anticomplementary.	Anticomplementary.	Not anticomplementary.	Anticomplementary.	Not anticomplementary.	Anticomplementary.	Not anticomplementary.	Anticomplementary.	Preparations without antigenic value.	Preparations without anticomplementary action.	Preparations with anticomplementary action.	Preparations without antigenic value.	Total.
Under 1 yr.	3	3		1			1				1	4	4		8
1 to 10 yr.				2							1		2	1	3
11 to 20 yr.					1	1						1	1		2
21 to 40 yr.	4	2	1	2	3	1	3	1				11	6		17
41 to 60 yr.	5		2		2	5	4	3	1		2	14	8	2	24
61 to 80 yr.	1			1	7	1	3				1	11	2	1	14
over 80 yr.			1					1				1	1		2
Total	13	5	4	6	13	8	11	5	1		4	42	24	4	70
	70														

5. THE ASSOCIATION BETWEEN THE AGE AND THE ANTIGENIC VALUE OF THE ACETON-INSOLUBLE FRACTIONS.

The foregoing analysis seems to show, in a general way, that the livers from individuals under twelve months of age yield lipoids of a higher antigenic value. With this exception no definite relation between the age and the antigenic power could be established. We suggest, however, that the possibilities of encountering pathological conditions leading to fatty degeneration of the liver are greater in adults. From this it would follow that the livers of older people are more likely to yield unserviceable lipoid fractions. Here the age is no more than a predisposition, and not the direct cause. The determining factor is, therefore, the pathological condition itself. The fact is worth recording that one of us (Noguchi) has on two occasions observed that, in spite of its normal appearance, the liver of a tubercular patient yielded a fraction which was distinctly hemolytic and only feebly antigenic.

STANDARD ANTIGEN FOR NOGUCHI'S METHOD.

A standard antigen for Noguchi's method for the diagnosis of syphilis may be prepared as follows: as a stock solution make an ethereal or alcoholic 3 per cent. solution of an aceton-insoluble fraction of the liver or heart. From this an emulsion is made by shaking one volume of the stock solution with nine volumes of a 0.9 per cent. sodium chloride solution. This emulsion is tested for its properties. If it is hemolytic or anticomplementary, in the dose of 0.4 cubic centimeter, it is unsuitable. When the emulsion is found to be non-hemolytic and non-anticomplementary, it is tested for its antigenic strength. If it produces complete inhibition of hemolysis with one unit of syphilitic antibody in doses of 0.02 cubic centimeter or less, it is suitable. In the fixation test 0.1 cubic centimeter of such an emulsion is to be used, thus employing more than five times the minimal antigen dose. So far as our experience goes, the use of several antigen doses does not cause a non-specific fixation and is not unduly sensitive.

The stock solution of antigen retains its properties for a long time. A solution prepared over a year ago has apparently undergone no change.

SUMMARY.

The liver tissues of man and certain animals (dogs, rabbits, guinea pigs, etc.) yield, upon alcoholic extraction, various substances which may be divided by their physical and chemical properties into several groups. While many substances are present in the alcoholic extract, the ones possessing antigenic properties are comparatively few. The latter are responsible for the antigenic properties exhibited by the whole alcoholic extract. The substances extracted with alcohol were fractionated into the following four groups.

(*a*) *Substances Insoluble in Ether and Hot Alcohol.*—These are chiefly proteins and salts. The proteins are probably the minute particles of larger molecules held in apparent suspension in alcohol until all other substances are removed. The water extracted from the tissues and admixed with alcohol is also an essential factor in extracting these particles in an alcoholic solution. The salts present are the usual physiological constituents of the liver, notably, sodium chloride. The quantity of these substances extracted with alcohol varies greatly with different specimens. Biologically considered, they are neither markedly hemolytic nor anticomplementary and possess no antigenic property for the Wassermann reaction. It is important, however, to note that the proteins bind complement when mixed with certain active human sera. For this reason a preparation of antigen containing this group of substances is unsuitable for use in combination with an *active* serum, and should, therefore, be rejected.

(*b*) *Substances Insoluble in Ether and Soluble in Hot Alcohol.*—This group contains soaps, cleavage products of proteins, and small amounts of the bile salts. Soaps and bile salts are very strongly hemolytic and are absolutely unfit for use as antigen. Moreover, their antigenic properties are very slight. It is best to eliminate this group of substances from the preparation of antigen. The quantity of the substances of this group extracted from different specimens of tissue is very variable.

(*c*) *Substances Soluble in Ether, Alcohol, and Aceton.*—In this group are found varying amounts of fatty acids, both saturated and unsaturated, some neutral fats, cholesterin and many unidentified lipoidal bodies. This group causes either hemolysis or inhibition of hemolysis. In other words, it is anticomplementary as well as hemolytic in the majority of preparations. At the same time, in some preparations it is, to a certain extent, antigenic. This great variation in the amounts of these substances in given extracts renders their presence in the antigen preparation undesirable. It is not denied, however, that, when added in adequate quantities, some of these substances may improve the activity of the antigenic lipoids.

(*d*) *Substances Insoluble in Aceton.*—This group of substances consists of phosphatids. The best known among them is, of course,

lecithin. Besides lecithin, however, there must be various other phosphatids present in this fraction. It will be noticed that the precipitate formed by mixing the ethereal solution with aceton contains a certain amount of lipoids insoluble in ether as well as in alcohol. Before the fractionation in aceton, all lipoids were soluble in ether or ethyl alcohol. Further analytical work on the nature of the phosphatids contained in this fraction is necessary. This fraction, in general, is more constant in amount in the various liver extracts. Biologically considered, it is the most important. It is usually non-hemolytic, frequently anticomplementary, but much more strongly antigenic than the other fractions. The antigenic strength varies with different preparations, being almost absent in the extracts derived from fatty livers. An aceton insoluble fraction may be strongly antigenic without any other auxiliary effects, or may be accompanied by an anticomplementary property. This fraction does not cause the so-called non-specific reaction with an active human serum. For these reasons it is recommended (as Noguchi has repeatedly done before) that the antigen should be selected from the aceton-insoluble fractions.

Iodine Value in Relation to Antigenic Value.—Speaking generally of the aceton-insoluble fraction, a high antigenic value is associated with a high iodine value of lipoids obtained from the liver and heart. This rule does not, however, apply to the corresponding fraction from the brain.

Standard Antigen.—The preparation of a standard antigen for Noguchi's method for the diagnosis of syphilis is given just before the summary. The directions will enable one to make a stock solution, from which, at any time, a suitable antigenic emulsion may be obtained.

VARIATIONS IN THE COMPLEMENT ACTIVITY AND FIXABILITY OF GUINEA PIG SERUM.[1]

By HIDEYO NOGUCHI and J. BRONFENBRENNER.

(*From the Laboratories of the Rockefeller Institute for Medical Research, New York.*)

Some time ago one of us (Noguchi) encountered a peculiar action in goat serum. This serum, while fresh and active, remained undeviated by the Wassermann reaction or by any combination of specific antigen and antibody. It was natural, therefore, to inquire whether a similar action might not be found in the sera of other species of animals or in the sera of individuals of a species in which the complement is generally capable of deviation.

Our present paper concerns itself with the quantitative determination of the complementary activity of various sera and their behavior towards the complement deviation phenomenon.

The most important and most widely studied of all complements is that of the guinea pig. Although there are some observations bearing upon the relative complementary activity of different specimens of this serum, there is no work in which quantitative estimations of complementary activity of a large number of specimens with simultaneous determinations on the fixability of each has been carried out. This has been done in our present studies.

We have also studied the complementary activity of the sera of several other species, such as beef, sheep, goat, pig, dog, cat, rabbit, rat, chicken, etc.[2] Some of these sera were tested for their fixability, our purpose being to see whether the complements of any of these could be used for the fixation test.

METHOD OF STUDY.

1. *The Titration of the Activity of Complement.*—The amboceptor derived from rabbits immunized with washed human corpuscles was used in all the

[1] Received for publication, September 21, 1910.

[2] The results with these sera will be published separately.

TABLE I.

Serial number of guinea pig sera.	Complement activity.							Fixability (20-hour-old serum).		
	Serum left in contact with clot for 20 hours (0° C.).			Serum left in contact with clot for 46 hours (0° C.).			Activity after 46 hours.	Amount of guinea pig serum fixed.	Number of complement units fixed.	Remarks on fixability.
	Hemolysis 100 per cent.	Hemolysis 50 per cent.	Hemolysis 25 per cent.	Hemolysis 100 per cent.	Hemolysis 50 per cent.	Hemolysis 25 per cent.				
1	0.025	0.012	0.006	0.025	0.012	0.006		0.135	5.4	Marked.
2	0.022	0.01	0.005	0.015	0.006	0.0035	Increased	0.12	5.45	Marked.
3	0.022	0.01	0.005	0.025	0.012	0.0055		0.09	4.09	Normal.
4	0.022	0.01	0.005	0.015	0.007	0.0035	Increased.	0.09	4.09	Normal.
5	0.022	0.01	0.005	0.015	0.007	0.004	Increased.	0.09	4.09	Normal.
6	0.022	0.01	0.005	0.022	0.009	0.005		0.147	6.68	High.
7	0.018	0.008	0.004	0.04	0.02	0.01	Marked decrease.	0.085	4.72	Normal.
8	0.018	0.008	0.005	0.022	0.01	0.005	Decreased.	0.09	5.	Normal.
9	0.022	0.009	0.005	0.022	0.01	0.005		0.09	4.09	Normal.
10	0.015	0.0065	0.004	0.013	0.006	0.003		0.09	6.	High.
11	0.017	0.0075	0.004	0.015	0.007	0.0035		0.09	5.3	Marked,
12	0.015	0.007	0.004	0.017	0.0075	0.0035		0.09	6.	High.
13	0.022	0.01	0.006	0.025	0.012	0.005		0.12	5.83	High.
14	0.02	0.008	0.004	0.015	0.006	0.003	Slight increase.	0.09	4.5	Normal.
15	0.022	0.01	0.006	0.015	0.006	0.003	Increased.	0.06	2.54	Highly refractory,
16	0.025	0.012	0.006	0.02	0.01	0.005	Slight increase.	0.09	3.6	Markedly refractory.
17	0.025	0.012	0.006	0.025	0.0121	0.006		0.15	6.	High.
18	0.02	0.01	0.005	0.018	0.008	0.004		0.09	4.5	Normal
19	0.022	0.01	0.005	0.02	0.01	0.005		0.085	3.86	Markedly refractory.
20	0.02	0.008	0.004	0.022	0.009	0.005	Slight decrease.	0.14	7.	High.
21	0.02	0.008	0.004	0.02	0.008	0.004		0.085	4.25	Normal.
22	0.02	0.008	0.004	0.018	0.0065	0.004	Slight increase.	0.09	4.5	Normal.
23	0.018	0.008	0.004	0.015	0.006	0.003	Slight increase.	0.09	5.	Normal.
24	0.02	0.008	0.004	0.022	0.01	0.006	Slight decrease.	0.09	4.5	Normal.
25	0.02	0.008	0.004	0.015	0.006	0.0035	Increased.	0.09	4.5	Normal.
26	0.022	0.009	0.005	0.015	0.006	0.003	Marked increase.	0.03	1.66	Highly refractory.

Serial number of guinea pig sera.	Complement activity.							Fixability (20-hour-old serum).		
	Serum left in contact with clot for 20 hours (0° C.).			Serum left in contact with clot for 46 hours (0° C.).			Activity after 46 hours.	Amount of guinea pig serum fixed.	Number of complement units fixed.	Remarks on fixability.
	Hemolysis 100 per cent.	Hemolysis 50 per cent.	Hemolysis 25 per cent.	Hemolysis 100 per cent.	Hemolysis 50 per cent.	Hemolysis 25 per cent.				
27	0.02	0.008	0.004	0.02	0.008	0.0045		0.09	4.5	Normal.
28	0.018	0.0075	0.004	0.015	0.006	0.004	Slight increase.	0.09	5.	Normal.
29	0.018	0.0075	0.004	0.015	0.006	0.0035	Slight increase.	0.09	5.	Normal.
30	0.035	0.015	0.0075	0.022	0.0075	0.004	Increased.	0.09	2.57	Highly refractory.
31	0.025	0.01	0.0045	0.015	0.006	0.003	Marked increase.	0.09	3.6	Markedly refractory.
32	0.02	0.008	0.004	0.022	0.01	0.006	Slight decrease.	0.15	7.5	High.
33	0.025	0.01	0.005	0.06	0.03	0.015	Marked decrease.	0.24	9.6	Unusual.
34	0.025	0.01	0.004	0.018	0.008	0.005	Increase.	0.	0.	Absolutely refractory.
35	0.025	0.01	0.004	0.022	0.0085	0.0045	Slight increase.	0.075	3.	Highly refractory.
36	0.02	0.008	0.004	0.022	0.009	0.005	Slight decrease.	0.09	4.5	Normal.
37	0.018	0.0075	0.004	0.018	0.007	0.0035		0.105	5.8	Marked.
38	0.027	0.012	0.005	0.04	0.02	0.01	Marked decrease.	0.12	4.44	Normal.
39	0.025	0.01	0.004	0.018	0.008	0.005	Marked increase.	0.12	4.8	Normal.
40	0.025	0.01	0.005	0.022	0.01	0.005	Slight increase.	0.09	3.6	Markedly refractory.
41	0.027	0.012	0.004	0.017	0.007	0.004	Marked increase.	0.09	3.33	Markedly refractory.
Averages	0.0216			0.0209				0.098	4.64	

hemolytic experiments. In addition to this, another amboceptor derived from a goat similarly immunized was employed in certain experiments recorded in the latter part of this paper. Unless otherwise stated, however, rabbit amboceptor is referred to.

The amboceptor was used in constant doses. For each tube 0.1 c.c. of a 10 per cent. suspension of washed human corpuscles was used. The complement to be titrated was added to a series of tubes in graduated quantities. The total volume of fluid in each tube was brought up to 1 c.c. by means of the addition of 0.9 per cent. salt solution. The tubes were placed in a water thermostat and incubated for two hours at 37° C.

2. *The Determination of the Fixability of Complement.*—In determining the fixation of complement, the system used was the one introduced by Noguchi for the diagnosis of syphilis. The amount of antihuman amboceptor (rabbit) was uniformly two units for each tube The quantity of washed corpuscles used was 0.1 c.c. of a 10 per cent. suspension. The quantity of complement varied according to the titers of the different sera. The amount of antigen emulsion was 0.1 c.c., which contained about four antigenic units. It was made from the aceton-insoluble fraction of lipoids from a human liver, according to Noguchi's method.[1] In doses of 0.45 c.c. it had no anticomplementary action. The serum was a mixture from several untreated cases of secondary syphilis and was inactivated before use. It had no inherent anticomplementary property in doses of 0.2 c.c., and in the fixation experiments the quantity employed was only one-tenth of this.

The determination of the amount of fixed complement was made by reading, according to the colorimetric method of Madsen, the degree of hemolysis present in a tube where an incomplete fixation had taken place, and then calculating the approximate amount of complement still free. As the exact amount of complement originally employed is known, it is an easy matter to calculate the amount of complement which had been fixed.

The results obtained are given in Table I. In this series of experiments forty-one guinea pigs were examined at the same time.

In studying the results summarized in Table I, several interesting facts may be noticed. It was rather unexpected to find that the complementary activity increased more or less after the sera remained in contact with the clot for forty-six hours, when compared with the activity of the same sera left in contact with the clot for twenty-hours after the bleeding. A few sera, however, became somewhat weaker under the same circumstances. The following analysis gives the results that were obtained.

The average titers for the first (twenty hours) and second (forty-six hours) determinations are nearly the same, being 0.0216 cubic centimeter for the first, and 0.0209 cubic centimeter for the

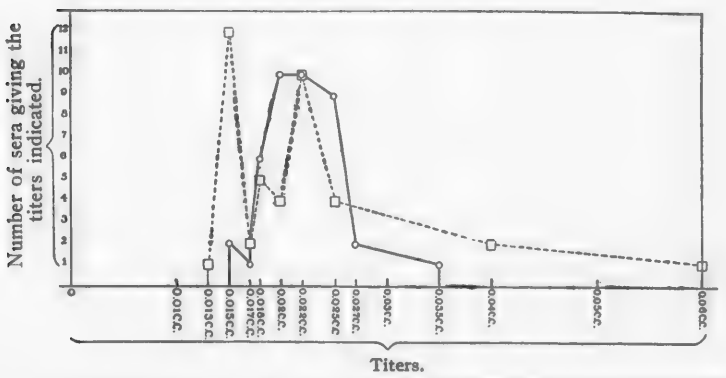
[1] Serum Diagnosis of Syphilis, 1st edition, Philadelphia, 1910, p. 71.

Comparable titers.	Number of sera examined. Determination after 20 hours.	Determination after 46 hours
0.013 c.c.	0	1
0.015 c.c.	2	12
0.017 c.c.	1	2
0.018 c.c.	6	5
0.02 c.c.	10	4
0.022 c.c.	10	10
0.025 c.c.	9	4
0.027 c.c.	2	0
0.03 c.c.	1	0
0.035 c.c.	0	0
0.04 c.c.	0	2
0.06 c.c.	0	1
	41	41

second. It will be noticed, also, that the differences between the highest and the lowest titers are much greater with the second determination. To insure greater uniformity in action, it is preferable to employ sera which have been left in contact with the clot for about twenty-four hours.

The titers of these forty-one sera are not absolute; they can be made higher by employing a larger quantity of amboceptor. We sought not absolute, but relative titers in our present experiments, and the following chart shows the frequency with which the same titers were obtained with different sera.

CHART 1.

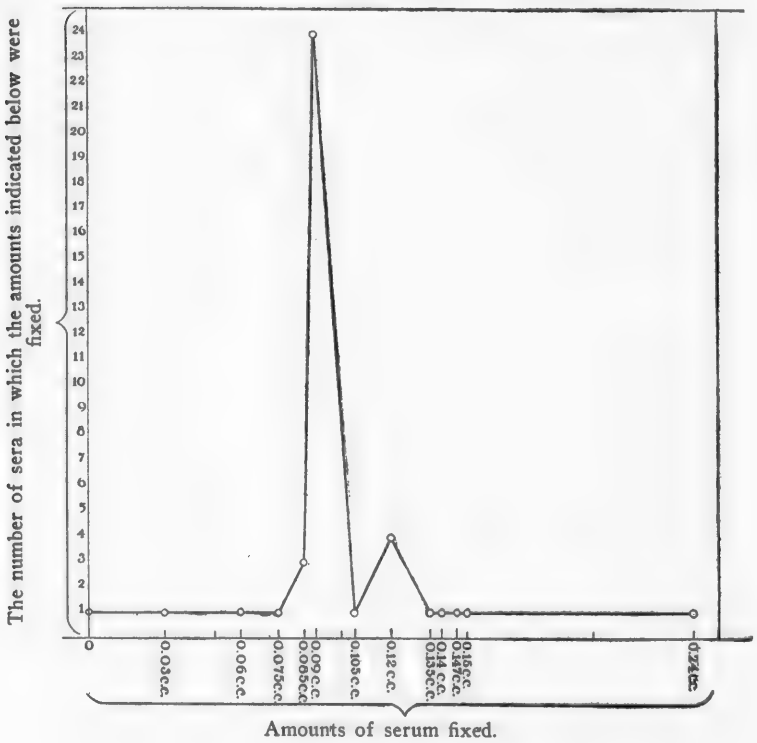


–O– Determination for the sera that remained in contact with the clot for 20 hours (0°C.)
–□– Determination for the sera that remained in contact with the clot for 46 hours (0°.C)

Let us now consider the relative susceptibility of different sera to the fixing phenomenon in the syphilis reaction. Here we have two

Amount of guinea pig serum fixed.	Number of specimens having the titers indicated.	Amount of guinea pig serum fixed.	Number of specimens having the titers indicated.
0.0 (non-fixable)	1	0.12 c.c.	4
0.03 c.c.	1	0.135 c.c.	1
0.06 c.c.	1	0.14 c.c.	1
0.075 c.c.	1	0.147 c.c.	1
0.085 c.c.	3	0.15 c.c.	1
0.09 c.c.	24	0.24 c.c.	1
0.105 c.c.	1		
			41

CHART 2.



problems. The first is to determine the absolute quantities of different sera fixed by given constant amounts of syphilitic serum and antigen; and the second is to determine the number of units of complement that have disappeared. The absolute amounts of sera fixed under the same conditions vary considerably with different specimens, but the variation in the majority of the specimens is slight.

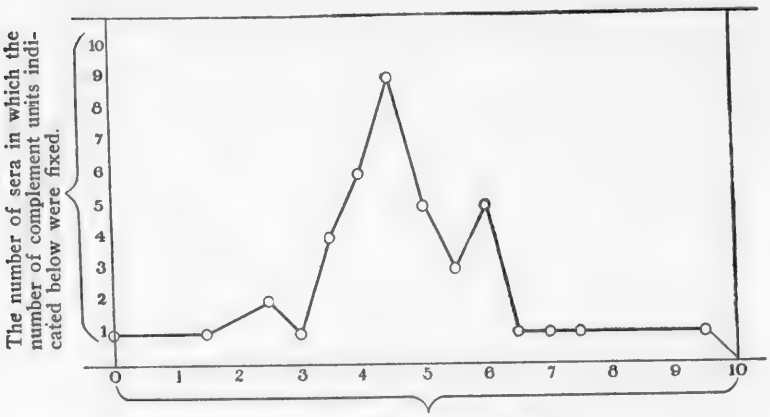
The contrast between the extremes in the fixation of guinea pig serum is most striking. The non-fixable serum (No. 34) showed no abnormal complementary activity, and the highest fixable serum (No. 33) had also the usual titer of activity, although the latter deteriorated with an unusual rapidity during forty-six hours. Another specimen (No. 38) had also shown a rapid deterioration, but there was no abnormality in its fixability.

Taking up the same subject from the standpoint of unity of complementary activity, the following statistics were obtained. In grouping the units, the fractions intermediate between two standard divisions were included in the nearest division. Several arbitrary zones of fixability may be created (see below) in order to enable us to gain a better idea of the occurrence of various degrees of fixability among the sera thus examined. In the zone of normal fixability

Number of units of complement fixed.		
0.	1	Zone of non-fixability, 1 specimen (2.4 per cent.).
1.5	1	Zone of inferior fixability, 8 specimens (19.5 per cent.).
2.	0	
2.5	2	
3.	1	
3.5	4	
4.	6	Zone of normal fixability, 20 specimens (48.7 per cent.).
4.5	9	
5.	5	
5.5	3	Zone of superfixability, 12 specimens (29.2 per cent.).
6.	5	
6.5	1	
7.	1	
7.5	1	
8.	0	
8.5	0	
9.	0	
9.5	1	
	41	

are included the specimens which varied from the average by 0.5 of a unit in either direction.

CHART 3.



The average amount of serum fixed was 0.098 cubic centimeter (see Table I, p. 71) and the average number of units fixed was 4.64 (see Table I, p. 71). In fact, the majority of sera possessed a susceptibility to fixation not far removed from the average.

SUMMARY.

The following conclusions may be drawn from the foregoing series of experiments. The complementary activity varies within a definite limit in different specimens of guinea pig serum. With sera which stood in contact with the clot for twenty hours, the strongest and weakest were in the ratio of 0.015 cubic centimeter to 0.04 cubic centimeter. The former was 2.66 times stronger than the latter. The variation observed with the same series of sera after forty-six hours was still more striking. The strongest was 0.013 cubic centimeter, and the weakest, 0.06 cubic centimeter, that is, the former was 4.6 times stronger than the latter. These findings agree with those made by Massol and Grysez.[4] The variations

[4] Massol and Grysez, *Compt. rend Soc. de biol.*, 1910, lxvii, 588.

were not so marked with the majority of sera. It is noteworthy that a large number of the sera gained in the complementary activity when remaining in contact with the clot for forty-six hours, while some sera became weakened during the same length of time.

The amount of serum *fixed* by given constant quantities of syphilitic serum and antigen varies much more markedly than the variations in their *complementary* activity. One serum failed altogether to be fixed. On the other hand, one sample of serum was so easily fixable that 0.24 cubic centimeter (corresponding to 9.6 complement units of this specimen) disappeared, while the average quantity fixed was only 0.098 cubic centimeter (corresponding to 4.64 complement units). The normal standard of fixability was shown in about 50 per cent. of the specimens examined. If the zone of normal fixability is enlarged in both directions by one unit, the percentage of normal fixability would become 65.8. There is no definite relationship between the complementary activity and the fixability of a given specimen of guinea pig serum.

The facts derived from our present experiments, especially in regard to the exceptions in the fixative quality of this serum, demand the utmost precaution from those intending to employ it for diagnostic purposes, as, for example, in the Wassermann reaction. No quantitative work is possible with the complement fixation reaction unless the experimenter is capable of determining the fixability of the serum in use. One of us (Noguchi) has long realized this source of error, and in order to reduce it he has advised the employment of a mixture of sera from more than two guinea pigs.

THE COMPARATIVE MERITS OF VARIOUS COMPLEMENTS AND AMBOCEPTORS IN THE SERUM DIAGNOSIS OF SYPHILIS.[1]

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When using a hemolytic antihuman amboceptor produced in the goat, one of us (Noguchi)[2] found that goat complement could not be deviated or fixed by the precipitate formed by mixing an extract of *Diplococcus intracellularis* (Weichselbaum) with antimeningococcic serum (Flexner-Jobling), or by mixing sera of man or different animals with their specific precipitins (rabbit), or egg-white with its specific precipitin. Under similar conditions, goat complement can not be deviated in Noguchi's method of the syphilis reaction. The amboceptor from the goat seemed to have a stronger affinity for the goat complement and liberated the complement after it had been fixed. Noguchi has also repeatedly shown, in the Bordet-Gengou phenomenon or in the syphilis reaction, that rabbit complement is less firmly fixed than guinea pig complement. As he was using a rabbit amboceptor, he could not determine whether the difficulty in fixing this complement was due to the use of an homologous amboceptor (from the same species) or to the inferior fixing qualities of the complement itself. In order to determine this point, it was necessary to prepare amboceptors from different species of animals. The preceding problem, together with others concerning the comparative merits of the complements of various species of animals in the complement fixation phenomenon, the Bordet-Gengou, and the Wassermann reactions, is considered in this communication. The questions just outlined include the determinations of the complementary activity of the sera of various

[1] Received for publication, September 21, 1910.

[2] *Proc. Soc. Exper. Biol. and Med.*, 1910, vii, 55.

species of animals with homologous and heterologous amboceptors. They also bring out the relative capacities of different species of animals for producing hemolytic amboceptor for human erythrocytes. Apart from the scientific interest, we intended also to search for a suitable species of the larger domestic animals whose complement or amboceptor would yield results as reliable as those obtained with guinea pig complement and rabbit amboceptor. If this should be accomplished, an economy would result.

PREPARATION OF ANTIHUMAN AMBOCEPTOR.

In our experiments the washed human erythrocytes suspended in 0.9 per cent. salt solution, corresponding with the original volume of the blood, was used for immunizing the animals. The varieties of animals used and the quantities of erythrocytes injected are given below:

Dog, subcutaneous injection,	20, 30, 30, 30 c.c.
Cat, intraperitoneal injection,	5, 10, 15, 20 c.c.
Rabbit, intraperitoneal injection	5, 10, 15, 20 c.c.
Guinea pig, intraperitoneal injection,	2, 3, 5, 7 c.c.
Rat, intraperitoneal injection,	1, 2, 3, 3 c.c.
Chicken, intraperitoneal injection,	3, 4, 5, 5 c.c.
Goat,[8] subcutaneous injection.	

With the exception of the goat, more than two animals of each of the species were employed, and several of each of the smaller animals. Injections were made four to five days apart, and the serum was collected on the ninth day after the last injection. These amboceptoric sera were inactivated at 56° C. for thirty minutes. After inactivation, the strength of amboceptor of each of the above animals was determined by titration with homologous and heterologous complement. The complements employed were from dog, cat, rabbit, guinea pig, rat, chicken, goat, pig, sheep, and ox. Owing to the small quantity of blood that could be obtained from the immunized rats, the amboceptor from this animal could not be titrated to the extent of the others.

The amount of complement of each species used was uniformly 0.05 cubic centimeter for each tube. The erythrocytic suspension

[8] This goat was given about twenty subcutaneous injections.

TABLE I.

	Rabbit amboceptor.				Guinea pig amboceptor.				Dog amboceptor.				Cat amboceptor.				Chicken amboceptor.			
	Complete hemolysis.	Moderate hemolysis.	Trace of hemolysis.	No hemolysis.	Complete hemolysis.	Moderate hemolysis.	Trace of hemolysis.	No hemolysis.	Complete hemolysis.	Moderate hemolysis.	Trace of hemolysis.	No hemolysis.	Complete hemolysis.	Moderate hemolysis.	Trace of hemolysis.	No hemolysis.	Complete hemolysis.	Moderate hemolysis.	Trace of hemolysis.	No hemolysis.
Rabbit complement	**0.005**	**0.003**	**0.0007**	**0.0003**	0.0015	0.0007	0.00035	0.00015	0.0025	0.001	0.0005		0.004	0.0025	0.005					0.05
Guinea pig complement	0.0025	0.0015	0.0005	0.0002	**0.001**	**0.0005**	**0.0002**	**0.0001**	0.025	0.015	0.001	0.0005	0.015	0.01	0.001	0.0005				0.05
Pig complement	0.04	0.015	0.0025	0.001	0.025	0.015	0.001	0.0005	0.015	0.01	0.0005	0.0002	0.015	0.01	0.001	0.0005	0.08	0.03	0.005	0.002
Sheep complement	0.05	0.025	0.005	0.0025	0.05	0.025	0.005	0.0025				0.05				0.05				0.05
Ox complement	0.045	0.025	0.005		0.04	0.02	0.005		0.04	0.02	0.005		0.025	0.015	0.0035	0.0007				0.05
Rat complement	0.01	0.005	0.001	0.0005	0.01	0.005	0.0015	0.0005		0.025	0.005	0.002		0.04	0.025	0.015				0.05
Goat complement	0.0025	0.0015	0.0005	0.0002	0.0025	0.001	0.0005	0.0002	0.001	0.0005	0.0002	0.0001	0.005	0.0025	0.0005					0.05
Chicken complement		0.07	0.01		0.01	0.005	0.001		0.05	0.03	0.002	0.001	0.07	0.03	0.005	0.002	**0.03**	**0.015**	**0.0005**	
Cat complement	0.0025	0.0015	0.0007	0.0004	0.0025	0.0015	0.0005	0.0002	0.025	0.015	0.0025	0.001	**0.004**	**0.001**	**0.0025**	**0.0005**				0.05
Dog complement	0.005	0.0025	0.0008	0.0004	0.005	0.0025	0.0005	0.0002	**0.025**	**0.015**	**0.0025**	**0.001**		0.035	0.015	0.01				0.05

TABLE II.

Species of animal furnishing amboceptor.	Arrangement of different complements according to their capacity to reactivate different antihuman amboceptors.								
Rabbit	Amboceptor titer 0.0015 with guinea pig complement goat complement cat complement	Amboceptor titer 0.0025 with dog complement	Amboceptor titer 0.003 with rabbit complement	Amboceptor titer 0.005 with rat complement	Amboceptor titer 0.015 with pig complement	Amboceptor titer 0.025 with ox complement sheep complement	Amboceptor titer 0.07 with chicken complement		
Guinea pig	Amboceptor titer 0.0005 with guinea pig complement	Amboceptor titer 0.0007 with rabbit complement	Amboceptor titer 0.001 with goat complement	Amboceptor titer 0.0015 with cat complement	Amboceptor titer 0.0025 with dog complement	Amboceptor titer 0.005 with chicken complement rat complement	Amboceptor titer 0.015 with pig complement	Amboceptor titer 0.02 with ox complement	Amboceptor titer 0.025 with sheep complement
Dog	Amboceptor titer 0.0005 with goat complement	Amboceptor titer 0.001 with rabbit complement	Amboceptor titer 0.01 with pig complement	Amboceptor titer 0.015 with guinea pig complement dog complement cat complement	Ambocepto titer 0.02 with ox complement	Amboceptor titer 0.025 with rat complement	Amboceptor titer 0.03 with chicken complement	Amboceptor had no action with sheep complement	
Cat	Amboceptor titer 0.001 with cat complement	Amboceptor titer 0.0025 with rabbit complement	Amboceptor titer 0.01 with goat complement	Amboceptor titer 0.015 with guinea pig complement pig complement	Amboceptor titer 0.03 with ox complement	Amboceptor titer 0.035 with chicken complement	Amboceptor titer 0.04 with dog complement rat complement	Amboceptor had no action with sheep complement	
Chicken	Amboceptor titer 0.015 with chicken complement	Amboceptor titer 0.03 with pig complement	Amboceptor had no action with rabbit, guinea pig, sheep, ox, rat, goat, cat, or dog complement.						

was employed in a dose of 0.1 cubic centimeter of a 10 per cent. suspension of washed human corpuscles. The total volume in each tube was made uniformly one cubic centimeter.

The above experiments revealed that a striking relation exists between the various complements and a given kind of amboceptor produced in different species of animals.

This is brought out in Table II.

With the amboceptors produced in guinea pig, cat, and chicken, the strongest action was obtained with their homologous complements. On the other hand, the amboceptors from the dog and rabbit acted more strongly with certain heterologous complements than with homologous complements. It was not the same heterologous complements, however, which showed this peculiarity. The strongest action with rabbit amboceptor was obtained with the complements of guinea pig, goat, cat, and dog. The complementary action of rabbit serum with dog amboceptor was, however, very much stronger than that of the dog complement with the amboceptor obtained from the dog. This dog amboceptor was thirty times more active in the presence of goat complement than in the presence of dog complement. Guinea pig complement, which has hitherto been considered universally suitable, is seen to be inferior to the complements of goat, rabbit, and pig when used with dog amboceptor, and it was without any complementary action for chicken amboceptor. The complements of ox and sheep, which were found to be quite suitable for goat amboceptor, are seen to be less suitable for the amboceptors derived from rabbit, guinea pig, dog, cat, and chicken. It is also remarkable that the sera of all mammals, except the pig, used in our experiments are devoid of any complementary action for chicken amboceptor, while chicken serum contains certain amounts of complement suitable for the amboceptors derived from those mammals.

Thus we have learned that the titers of an amboceptor may vary considerably according to the varieties of sera employed as complement. That the variations revealed in the titration of a given amboceptor are due to the variations in the complement content of the serum used as complement, can be further demonstrated by determining the limit of activity of the sera (serving as complement)

in the presence of a constant amount of amboceptor. In the following table is shown the comparative activity of different sera as complement. Rabbit amboceptor (in excess) was employed in this experiment.

TABLE III.

	Titration of sera as complement with antihuman rabbit amboceptor.									
	Guinea pig complement.	Cat complement.	Goat complement.	Dog complement.	Rabbit complement.	Rat complement.	Pig complement.	Ox complement.	Sheep complement.	Chicken complement.
Quantity for complete hemolysis	0.006	0.006	0.006	0.01	0.015	0.02	0.05	0.07	0.1	
Quantity for moderate hemolysis	0.003	0.003	0.003	0.005	0.007	0.01	0.03	0.035	0.05	0.07
Quantity for trace of hemolysis	0.002	0.002	0.002	0.003	0.004	0.005	0.015	0.015	0.02	0.02
Quantity for no hemolysis	0.001	0.001	0.001	0.0015	0.002	0.003	0.007	0.008	0.01	0.01

Table III shows that the titration of the complementary activity of the sera of guinea pig, cat, and dog is about twice as strong as that of the rabbit for rabbit amboceptor. In this respect sheep serum was about sixteen times weaker, and chicken serum was too weak to produce complete hemolysis.

We have also studied the complementary activity of various sera for the amboceptors derived from heterologous as well as from homologous species. Our results are given in Table IV.

This series of experiments demonstrates three interesting facts: (1) the preference of certain amboceptors for their homologous complements (chicken, guinea pig, goat); (2) the inferiority of certain complements for homologous amboceptors (rat); and (3) the increase of hemolytic activity of complements in general by the use of a larger amount of the amboceptor. The inferiority of homologous complements to certain heterologous complements has already been described with the sera derived from rabbits and dogs (see Table II). That cat amboceptor is more powerful with its homologous complement is shown in the same table. Thus, we find that the amboceptors derived from chicken, guinea pig, goat, and cat are stronger in the presence of their homologous complements,

TABLE IV.

Quantity of complement.	Rabbit complement.		Dog complement.		Cat complement.		Chicken complement.	Pig complement.	Pig complement.	Rat complement.	Guinea pig complement.		Guinea pig complement.		Goat complement.		Goat complement.	
	Rabbit amboceptor.		Dog amboceptor.		Cat amboceptor.		Chicken amboceptor.	Chicken amboceptor	Rat amboceptor.	Rat amboceptor.	Guinea pig amboceptor.		Goat amboceptor.		Guinea pig amboceptor.		Goat amboceptor.	
	(0.025)	(0.0025)	(0.025)	(0.0025)	(0.025)	(0.0025)	(0.1)	(0.05)	(0.1)	(0.1)	(0.025)	(0.002)	(0.03)	(0.005)	(0.025)	(0.0025)	(0.03)	(0.005)
0,1	C.H.	C.H.	Mch.H.	Mch.H.	C.H.	C.H.	C.H.	C.H.	C.H.	Sl.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
0.05	C.H.	Md.H.	Tr.H.	Tr.H.	C.H.	C.H.	C.H.	Mch.H.	C.H.	No H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
0.035	C.H.	Sl.H.	Tr.H.	No H.	C.H.	Sl.H.	Sl.H.	Tr.H.	C.H.		C.H.	C.H.	C.H.	Tr.H.	C.H.	C.H.	C.H.	C.H.
0.02	C.H.	Tr.H.	No H.		C.H.	No H.	No H.	No H.	Mch.H.		C.H.	No H.	C.H.	No H.	C.H.	Md.H.	C.H.	C.H.
0.01	C.H.	Tr.H.			Mch.H.				Tr.H.		C.H.		Mch.H.		Mch.H.	No H.	C.H.	Sl.H.
0.005	C.H.	No H.			Tr.H.				No H.		C.H.		Sl.H.		No H.		Md.H.	No H.
0.002	Md.H.				No H.						Mch.H.		Tr.H.				Tr.H.	
0.001	No H.										No H.		No H.				No H.	

C.H. = complete hemolysis; Mch.H. = much hemolysis; Md.H. = moderate hemolysis; Sl.H. = slight hemolysis; Tr.H. = trace of hemolysis.

while those from rabbit, dog, and rat are stronger in the presence of certain suitable heterologous complements.

After studying the complex inter-relation of various complements and amboceptors, we took up the further study of a few complements. The sera of pig, sheep, and ox were studied especially because we hoped to be able to utilize them as complement, for they may be obtained in large quantity from any abattoir. We soon discovered, however, that they were quite unsuitable for the syphilis reaction. In the first place, ox serum often contains a variable and sometimes considerable amount of natural amboceptor for human erythrocytes, and for this reason we did not pursue the study of this serum.

The sera of pig and sheep are usually devoid of the natural anti-human amboceptor. They were examined for their complementary activity for rabbit amboceptor. The results are shown in Tables V and VI.

The striking feature of the sera of these two animals is the rapid disappearance of their complementary activity. It is more marked with pig serum. Both varieties of serum became so weak after forty-eight hours (0° C.) that they no longer produced complete hemolysis in a quantity as large as 0.1 cubic centimeter. Guinea pig serum, under the same conditions, showed practically no diminution during the same period of preservation. The rapid deterioration of both pig and sheep serum renders them unreliable because of the constantly decreasing activity of complement. The rate of deterioration is also quite irregular with different samples of the same variety of serum. Thus, in point of reliability and activity, they are far inferior to the serum of the guinea pig and are not recommended for use in the syphilis reaction.

CONCERNING THE USE OF VARIOUS SERA AS COMPLEMENT IN THE SERUM REACTION OF SYPHILIS.

In deciding whether or not the serum of a given animal is suitable as complement in the complement deviation test, we have to consider two essential properties of the serum. The first is, of course, its complementary activity, and the second is its susceptibility to fixation. We have already studied in detail the comparative com-

TABLE V.

Pig Serum.

Serial number of pigs.	Titration with antihuman rabbit amboceptor, 0.01 c.c.									Titrated for natural antihuman amboceptor.	
	4 hours old.			24 hours old.			48 hours old.			4 hours old. 0.2	Inactivated at 56° C. 0.2
	0.1	0.05	0.025	0.1	0.05	0.025	0.1	0.05	0.025	Active.	+ 0.05 guinea pig serum.
No. 1.....	C.H.	C.H.	C.H.	C.H.	Mch.H.	Sl.H.	Sl.H.	Tr.H.	No H.	No H.	No H.
No. 2.....	C.H.	C.H.	C.H.	C.H.	C.H.	Mch.H.	Sl.H.	Tr.H.	No H.	No H.	No H.
No. 3.....	C.H.	C.H.	C.H.	C.H.	C.H.	Mch.H.	Md.H.	Tr.H.	No H.	No H.	No H.
No. 4.....	C.H.	C.H.	C.H.	C.H.	Mch.H.	Sl.H.	Sl.H.	No H.	No H.	No H.	No H.
No. 5.....	C.H.	C.H.	C.H.	C.H.	C.H.	Mch.H.	Mch.H.	Tr.H.	No H.	No H.	No H.
No. 6.....	C.H.	C.H.	C.H.	C.H.	C.H.	Sl.H.	Mch.H.	Tr.H.	No H.	No H.	No H.
No. 7.....	C.H.	C.H.	C.H.	C.H.	Mch.H.	Mch.H.	Sl.H.	No H.	No H.	No H.	No H.
No. 8.....	C.H.	C.H.	C.H.	C.H.	Mch.H.	Sl.H.	Sl.H.	No H.	No H.	No H.	No H.
No. 9.....	C.H.	C.H.	C.H.	Mch.H.	Mch.H.	Sl.H.	Sl.H.	No H.	No H.	No H.	No H.
No. 10.....	C.H.	C.H.	C.H.	C.H.	Sl.H.	Tr.H.	Sl.H.	No H.	No H.	No H.	No H.
Guinea pig serum. (control)	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.		

TABLE VI.

Sheep Serum.

Serial number of sheep.	Titration with antihuman rabbit amboceptor, 0.01 c.c.									Titrated for natural antihuman amboceptor.	
	4 hours old.			24 hours old.			48 hours old.			4 hours old, 0.2	Inactivated at 56° C. 0.2
	0.1	0.05	0.025	0.1	0.05	0.025	0.1	0.05	0.025	Active.	+ 0.05 guinea pig serum.
No. 1	C.H.	Almost C.H.	Mch.H.	C.H.	Mch.H.	No H.	Mch.H.	Tr.H.	No H.	No H.	Tr.H.
No. 2	C.H.	Almost C.H.	Mch.H.	Almost C.H.	Tr.H.	No H.	Sl.H.	No H.	No H.	No H.	No H.
No. 3	C.H.	Almost C.H.	Mch.H.	C.H.	Sl.H.	No H.	Almost C.H.	No H.	No H.	No H.	No H.
No. 4	C.H.	C.H.	Mch.H.	C.H.	Almost C.H.	Sl.H.	Mch.H.	Sl.H.	Tr.H.	Tr.H.	Sl.H.
No. 5	C.H.	C.H.	Mch.H.	C.H.	Almost C.H.	No H.	C.H.	Tr.H.	No H.	No H.	No H.
No. 6	C.H.	C.H.	Mch.H.	Almost C.H.	Almost C.H.	Tr.H.	Sl.H.	No H.	No H.	No H.	No H.
No. 7	C.H.	C.H.	Mch.H.	C.H.	Mch.H.	No H.	Md.H.	Tr.H.	No H.	No H.	Tr.H.
No. 8	C.H.	C.H.	Mch.H.	C.H.	Almost C.H.	Tr.H.	C.H.	Almost C.H.	Tr.H.	Tr.H.	Sl.H.
Guinea pig serum (control)	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.		

plementary activities of the sera of various species of animals. We propose to report here, therefore, the results which we have obtained with regard to their comparative fixability.

As mentioned at the beginning of this article, Noguchi long ago showed that the complement of goat serum remains undeviated by specific precipitates or by the syphilis reaction. This phenomenon was decidedly more pronounced when the goat furnished both complement and amboceptor. He has also demonstrated that rabbit serum is less sensitive to fixation than guinea pig serum. Hence he suspected that the amboceptor might have such a strong affinity toward its homologous complement as to reverse partially the process of fixation. The question of whether or not there is such a relation between the source of amboceptor and the fixability of complements was left unsettled. This was one of the problems which we set out to solve, and our results will now be given.

The fixation test was carried out with Noguchi's system in the usual way. The positive serum was from a case of untreated secondary syphilis, and, in the quantity of 0.002 cubic centimeter, was capable of fixing completely 0.04 cubic centimeter of guinea pig serum. In the following experiments, 0.02 cubic centimeter of the syphilitic serum was used for each tube.

TABLE VII.

	Complement.				
	Guinea pig 0.04 (2 units).	Rabbit No. 1 0.08 (2 units).	Rabbit No. 2 0.08 (2 units).	Goat No. 1 0.04 (2 units).	Goat No. 2 0.04 (2 units).
Guinea pig amboceptor (2 units)	No hemolysis	Partial hemolysis (no hemolysis for some time).	Partial hemolysis (no hemolysis for some time).	Complete hemolysis (much delay).	Complete hemolysis (much delay).
Rabbit amboceptor (2 units).	No hemolysis	Partial hemolysis (no hemolysis for some time).	Partial hemolysis (no hemolysis for some time).	Complete hemolysis (much delay).	Complete hemolysis (much delay).
Goat amboceptor (2 units).	Slight hemolysis (no hemolysis for some hours).	Much hemolysis (no hemolysis for some time).	Much hemolysis (no hemolysis for some time).	Complete hemolysis (slight delay).	Complete hemolysis (slight delay).

The experiments given above show that the complement contained in goat serum is totally unfixable. There was some delay in commencing hemolysis, but it was finally completed within a few hours. The complement contained in rabbit serum showed the peculiarity that hemolysis commenced after six hours or so, and after about twenty-four hours a moderate amount of hemolysis was observed. This is most striking when compared with the complete and lasting inhibition of hemolysis in the case of guinea pig complement. The use of goat amboceptor, however, has caused more or less hemolysis even with guinea pig complement. Experiments also show that when the complements are fixable the homologous amboceptor and complements do not influence the fixation phenomenon. It is difficult to explain why the fixation of guinea pig complement is imperfect when tested by goat amboceptor. It may be due to the presence in guinea pig serum of a non-fixable complement, which, while quite active with the goat amboceptor, is inactive with the amboceptors from rabbit and guinea pig. Now, with regard to the state of non-fixation of the complement of goat serum, there are two possibilities. The first is its insensitiveness to fixation. The delay in commencing hemolysis may be accounted for by assuming that the quantity of complement still free in the mixture was considerably reduced by a partial fixation. The second is the gradual liberation of complement already fixed through the introduction of the goat amboceptor. Apart from these hypothetical considerations, the above experiments showed that rabbit and goat complement and goat amboceptor are unsuitable in deviation tests.

We have determined the fixability of the complements contained in the sera of pigs and sheep. Pig complement was fixable, while that of sheep was somewhat less so. But, as has already been pointed out, their complementary activity is so feeble that, in order to employ two units, their absolute quantities may reach 0.1 cubic centimeter or even exceed this. If the fixation were *selectively* directed to the complement, an accurate result could always be secured by employing any quantity in which two activity-units existed. But as we shall describe elsewhere, the fixation is not selectively directed to the complement. The presence of non-complementary protein constituents may, therefore, interfere with the

fixation of complement. From this it will be easy to understand why we must avoid weak sera, for in these the quantity which contains a single unit of complement is too large.

CONCLUSIONS.

1. The maximum activity of an antihuman hemolytic amboceptor may be obtained by employing the homologous or heterologous complement, according to the variable relations existing between the species furnishing the amboceptor and the one supplying the complement. Thus, some amboceptors are best reactivated by the complement of the same species, while others may act most strongly when reactivated with the complements of certain suitable heterologous species.

2. From the above it is clear that the complementary activity of a given serum may be very variable according to the varieties of amboceptors employed. In expressing the complementary activity of a serum, the species of the host of the amboceptor must always be stated. Thus, one serum may have many different complementary titers according to the amboceptors used. A similar variation in the titers of the amboceptors occurs when a variety of complements are employed.

3. Certain species of animals (pig and sheep) yield sera which are comparatively poor in reactivating most varieties of antihuman amboceptors. The complements of these species deteriorate rapidly.

4. The serum of chicken contained but little complement for the amboceptors derived from the mammalia, while the amboceptor from the chicken was only poorly, or not at all, reactivable by the complements contained in the mammalian sera. The serum of pig was the only variety which reactivated this amboceptor in a fair degree.

5. For the fixation tests guinea pig complement is the most favorable. This complement is also the most active and durable of those which have been studied. The complements of pig and sheep are quite fixable, but their weakness and rapid deterioration render them unsuitable for fixation purposes. Rabbit complement is quite active but is not easily fixable. Goat complement is, as already

stated, difficult to fix, in spite of its strong complementary activity. The other complements are unsuitable because of their feeble complementary activity.

6. For fixation tests the antihuman amboceptors produced in the rabbit and guinea pig are suitable. They are, moreover, very active and do not cause the phenomenon of non-fixation. The amboceptors from other animals are unsuitable, as we cannot find a complement which strongly reactivates them. The amboceptor from the goat is unsuitable because of the danger of masking the fixation phenomenon by subsequent hemolysis.

7. In summing up, we arrive at the conclusion that the rabbit is the best animal for producing antihuman amboceptor, and the guinea pig for supplying complement. The guinea pig produces a good amboceptor, but its small size renders it second in choice.

So far, no other animals have been found useful for the fixation experiments.

THE INTERFERENCE OF INACTIVE SERUM AND EGG-WHITE IN THE PHENOMENON OF COMPLEMENT FIXATION.[1]

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Is the fixative action of a specific antigen-antibody combination directed only towards the serum constituents known as complement or towards any protein whose physico-chemical properties with regard to the fixation phenomenon are similar to complement? This question needs experimental determination, as the complementary activity of a given specimen of serum has no constant quantitative relationship to the amount of other non-complementary protein constituents contained in the same serum.

If the fixation were directed exclusively towards the active complementary constituents, we could measure quantitatively the fixing capacity of the combination of a given specific antigen and antibody by simply estimating the units of complement fixed. On the other hand, if the fixation is also directed towards the other indifferent proteins simultaneously present in the complement-containing serum, the quantitative estimation of the fixative capacity of the antigen-antibody combination becomes complicated; we cannot measure it by the units of complement fixed, for the fixation is also shared by the other serum components whose exact amounts cannot be ascertained by this method. A great miscalculation of the real fixing capacity must inevitably result when one uses as indicator the complementary activity alone. This source of error is steadily increased by the gradual disappearance of the complementary activity from the serum, while the amount of the other non-complementary constituents not only remains undiminished, but is increased by the gradual conversion of the active complement into an

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inactive complementoid. Before this fundamental relation is established, any attempt to estimate complement fixation quantitatively is premature.

In this communication we present the results which we obtained in studying the effects of various non-complementary proteins upon the complement fixation phenomenon, including the syphilis reaction. In our study we employed, as usual, the antihuman hemolytic system recommended by Noguchi. Every factor concerned in this system is under perfect quantitative control.

THE SYPHILIS REACTION.

In this series the effects of various inactivated or spontaneously deteriorated sera and egg-white were studied. Varying quantities of each serum and of egg-white, in a twenty per cent. dilution in 0.9 per cent. salt solution, were added under three different conditions to the fixing mixture of syphilitic serum and antigen (acetone-insoluble fraction of lipoid from human liver). They were added and incubated (1) before the addition of complement; (2) at the same time as the complement; and (3) after the complement had been fixed. Some tubes without any serum or egg-white were, of course, provided as controls. The amount of syphilitic serum used was 0.02 cubic centimeter for each tube, and this amount was capable of fixing 0.08 cubic centimeter of guinea pig complement in the presence of 0.1 cubic centimeter (four units) of the antigen emulsion. In the case of non-anticomplementary sera, the amount of complement used was uniformly 0.04 cubic centimeter of fresh guinea pig serum. On the other hand, certain deteriorated sera showed a more or less anticomplementary property. This had to be overcome by adding, in addition to the 0.04 cubic centimeter of complement, the exact amount of active complement (guinea pig serum) necessary to neutralize each such specimen. The total volume in each tube was made equal to one cubic centimeter. The incubation period was one hour at 37° C. The first and third series in Table I were twice incubated, as this was required by the nature of the experiments. Then 0.1 cubic centimeter of a 10 per cent. suspension of washed human corpuscles and two units of anti-

TABLE I.

	1st series. Inactive serum added before complement.			2d series. Inactive serum and complement introduced at same time.			3rd series. Complement added before inactive serum.		
	0.1	0.05	0.03	0.1	0.05	0.03	0.1	0.05	0.03
Guinea pig serum No. 1, inactivated by age. . .	C.H.	Mch.H.	Sl.H.	Mch.H.	Md.H.	Tr.H.	No H.	No H.	No H.
Guinea pig serum No. 2, inactivated by age. . .	C.H.	Mch.H.	Sl.H.	Mch.H.	Md.H.	Tr.H.	No H.	No H.	No H.
Guinea pig serum No. 3, inactivated, 56°C. . . .	C.H.	Md.H.	Sl.H.	Mch.H.	Md.H.	Tr.H.	No H.	No H.	No H.
Guinea pig serum No. 4, inactivated, 56°C. . . .	C.H.	Md.H.	Sl.H.	Mch.H.	Md.H.	Tr.H.	No H.	No H.	No H.
Human serum No. 1, inactivated by age.	Sl.H.	No H.	No H.	Tr.H.	No H,	No H.	No H.	No H.	No H.
Human serum No. 2, inactivated by age.	Mch.H.	Sl.H.	No H.	Sl.H.	No H.	No H.	No H.	No H.	No H.
Human serum No. 3, inactivated by age.	No H.	No H.	No H.	No H.	No H.	No H.	No H.	No H.	No H.
Human serum No. 4, inactivated, 56°C.	C.H.	Mch.H.	Sl.H.	Mch.H.	Sl.H.	No H.	No H.	No H.	No H.
Human serum No. 5, inactivated, 56°C.	Mch.H.	Sl.H.	No H.	Md.H.	Sl.H.	No H.	No H.	No H.	No H.
Sheep serum No. 1, inactivated by age	C.H.			Mch.H.			No H.		
Sheep serum No. 2, inactivated 56°C.	C.H.			Mch.H.			No H.		
Pig serum, inactivated by age.	C.H.			Mch.H.			No H.		
Horse serum, inactivated, 56°C.	C.H.			Mch.H.			No H.		
Cat serum, inactivated, 56°C.	C.H.			Mch.H.			No H.		
Dog serum, inactivated, 56°C.	Mch.H.	Sl.H.	No H.	Sl.H.	No H.	No H.	No H.		
Goat serum No. 1, inactivated by age.	Sl.H.	No H.	No H.	No.H.	No H.	No H.	No H.		
Goat serum No. 2, inactivated, 56°C.	Mch.H.	Sl.H.	No H.	Md.H.	Sl.H.	No H.	No H.		
Chicken serum, inactivated by age.	C.H.	Mch.H.	Sl.H.	Mch.H.	Sl.H.	No H.	No H.		
Chicken serum, inactivated, 56°C.	C.H.	Mch.H.	No H.	Md.H.	Tr.H.	No H.	No H.		
Egg-white (20%), unheated	C.H.	Almost C.H.	Md.H.	Almost C.H.	Mch.H.	Sl.H.	No H.	No H.	No H.
Egg-white (20%), heated to 56°C.	C.H.	Mch.H.	Sl.H.	Mch.H.	Sl.H.	No H.	No H.	No H.	No H.

human amboceptor (rabbit) were added to all tubes, mixed well, and incubated for two hours at 37° C. The results are given in Table I.

The foregoing experiments demonstrate several important points. The first series shows that the addition of various animal sera, from which the complementary property had disappeared either by a spontaneous deterioration or by an artificial inactivation, to the fixing mixture of syphilitic serum and antigen, saturated completely the latter's fixing capacity so that it was no longer capable of fixing the complement of fresh guinea pig serum subsequently introduced. Exceptions were encountered with human and goat sera, which behaved inconstantly. While some of the human sera interfered with the fixation to the same degree as that of most animal sera, others showed but little interference. The serum of the goat was also comparatively less interfering. The most remarkable fact in the series is that the egg-white acted in the same way as most animal sera.

In the second series we observe somewhat less interference of fixation by the sera and egg-white. This is not at all surprising, because of their simultaneous addition with the complement, under which condition the fixing had been directed to both. In the third series there was no hemolysis. It is evident that the subsequent addition of these substances had no reversing effect upon the complement, which had been fixed before they were introduced.

The deductions which may be drawn from these observations are that the fixing capacity of the syphilitic serum and antigen can be saturated not only by active complement-containing sera or inactive sera, but also by an apparently indifferent suspension of proteins (egg-white), so long as their physico-chemical properties are the same. It also shows how erroneous it is to estimate the fixing capacity by the estimation of one biological property (complementary) without reference to the ratio of this biological activity to the other components of the serum serving as complement. For quantitative work with the complement fixation test, the establishment of a standard ratio between the complementary unit and the volume of the serum, becomes essential. In addition to this,

the relation of the fixability of the sera of different species must be considered.

The Complement Fixation by Specific Precipitate (Bordet-Gengou Phenomenon).—Parallel series of experiments with the foregoing were also made with the precipitate produced by the anti-meningococcic horse serum (Flexner and Jobling) and meningococcic extract.

The results obtained were in perfect harmony with those of the syphilis reaction just described; hence protocols are not called for.

The fixability of these sera and egg-white diminished progressively at temperatures above 56° C., and disappeared at 85° C.

Alcohol, which coagulates proteins, removes the interfering property of sera and egg-white.

CONCLUSIONS.

The fixing property of a specific precipitate and of syphilitic serum in the presence of certain antigenic lipoids, can be removed by adding certain non-complementary proteins of blood serum or hen's egg.

This disappearance of the complementary activity in the syphilis reaction, as well as in the true Bordet-Gengou reaction, is a phenomenon which incidentally accompanies the fixation of certain serum constituents, some of which possess a complementary activity. The presence or absence of the complementary property in these protein components does not influence fixation. Whether the disappearance of the complementary activity during the phenomenon of so-called fixation is due to a mechanical precipitation of the molecules through absorption or whether it is due to a physico-chemical alteration of the active molecules, is unknown. It is more probable that a chemical interaction takes place in the case of the syphilis reaction. Certain sera, for example, those derived from man and goat, show a low fixability.

It is interesting to note that the fixability is gradually diminished when these sera and egg-white are heated to a temperature above 56° C., and totally disappears at 85° C. The coagulation of proteins with absolute alcohol or by boiling, destroys their interfering property.

The fact that the fixation is not selectively directed towards complement, has a very important meaning for exact serology. The one-sided accuracy as to the complementary unity is no longer sufficient for quantitative work. Both the complementary and the volumetric unity of a serum serving as the source of complement should be taken into consideration. Besides, the fixability of the sera of various species of animals must also be considered.

From these facts a formula may be derived for deciding the degree of suitableness of a serum.

$$X = K\frac{P}{V}$$

X is the degree of suitableness; K, the species constant for the fixability; P, the complementary activity; and V, the volume of serum. It will be seen that the suitableness is proportional to the fixability constant and the complementary unity, and inversely proportional to the volume of serum employed.

As to what species yields the largest value for X, we refer the reader to our studies published elsewhere.[2]

[2] (1) Variations in the Complement Activity and Fixability of Guinea pig Serum; (2) Comparative Merits of Various Complements and Amboceptors in the Serum Diagnosis of Syphilis, this number of the *Journal of Experimental Medicine.*

A HISTOLOGICAL STUDY OF THE SKIN LESIONS OF PELLAGRA.[1]

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PLATE V.

Although, during the past decade in Europe and more recently in this country, pellagra has been the subject of careful study, the pathological phenomena have received but little attention. Certain investigators, working upon the hypothesis that the disease is due to the action of a toxine produced by some of the fungi ingested along with food stuffs, have made an extensive study of the metabolic changes in pellagra. These studies, together with empirical methods of treatment and prophylaxis and close observation of the results of these, have undoubtedly resulted in an advance in our knowledge of the disease. At present, however, it may be said that no constant pathologic lesions have been accepted as characteristic of the disease.

Clinically the disease is recognized by a syndrome of symptoms which may be classed as nervous or functional, trophic, and, to a less marked extent, organic. To many observers one of the most helpful signs in the diagnosis of the condition is the presence of skin lesions. All writers are more or less impressed by the fact that the appearance of the lesions varies markedly in different cases and, in general, comparatively little effort has been made to correlate the diverse manifestations. Dyer (1), in particular, speaks of the diversity in the characteristics of the lesions of the skin. He says: "The cases seen by me had certain points in common, but were sufficiently dissimilar to suggest the probability that the skin evidences of pellagra are more apt to be the direct reflections of the associated etiological factors than a separate integral symptom."

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He does not, however, note what associated factors he considers potent in the production of the lesions in the different parts of the body.

The resemblance of the gross skin changes in pellagra to those of erythema solare, have been noted by several observers, especially as the affected parts in pellagra are usually those which are either exposed to light or are less sufficiently covered than the remaining skin surface. The esssential changes in the skin which bring about the clinical appearance of erythema, bullæ formation, and, finally, pigmentation and scaling, has received practically no attention. If it is possible to determine that the different phenomena are the result of essentially the same pathologic process, a decided advance will have been made. If, further, it can be shown that the lesions in different parts of the body are in similar tissues, the factor or factors causing these lesions remain to be investigated.

The presence of cutaneous lesions upon the arms, legs, or other parts of the body have been looked upon as essential phenomena of the malady. If, therefore, it can be shown that their character and situation is more or less accidental, and that their absence or severity depends as much upon secondary factors as upon the pellagrous process itself, the clinical diagnosis will be facilitated. In the explanation, too, of the cutaneous changes, a clue will be given as to the nature of lesions in the buccal, intestinal, and other mucous membranes.

The skin lesions in pellagra were first described by Casel (2) in 1735. Raymond (3) drew attention to the occasional rapid appearance of the skin lesions in patients already suffering from other manifestations of the disease.

Merck (4) describes the cutaneous lesion as a more or less diffuse erythema which subsequently became pigmented and scaly, the pigmentation and hyperkeratosis continuing as characteristic of the disease. He says that the tissues are frequently swollen as the result of an excess of blood and serum in the derma and, in consequence, wrinkling may occur subsequently. The lesions usually begin at the base of the fingers and upon the dorsum of the foot. In the former situation they spread and form a pigmented, scaly, triangular area, the apex of which is at the olecranon and the

base at the metacarpal-phalangeal joints. In a similar manner the lesions commencing upon the foot may spread toward the knee. Occasionally lesions appear on the face, around the neck, and down the front of the sternum (the so-called " Casel's neck-tie "). Other parts of the body, for instance, the buccal mucosa and genitals, are occasionally affected, but in these situations the lesions are not analogous to the dermatitis here described. In the severer forms vesicles or bullæ are present early in the process.

Babes and Sion (5) were the first to study microscopically the lesions of the skin. They describe a slight serous exudation and leucocytes in the erythematous stage, together with the presence of peculiar homogeneous metachromatic masses of what they considered to be coagulated albumin. These observers did not note changes in the nerves. In the stage of desquamation and hyperkeratosis, the papillae of the corium contained lymphoid and plasma cells, and a few mast cells. The sweat glands were atrophied. They consider that the thickening of the skin is the result of increased blood supply. They mention, also, the swollen appearance of degenerating elastic tissue fibrils in the superficial layers of the derma. In the cases which ulcerate, the phenomena they describe are the same as those observed in ordinary chronic ulcerations. Harris (6) confirmed, in general, the findings of Merck and Babes and Sion, stating, however, than an erythema does not always occur.

Roher (7) considers that pellagra is a granulomatous disease, basing his view upon the occurrence of collections of spindle-shaped and small round cells surrounding a central necrotic area. These nodules, he considers, are usually present in the vessels in the lungs and brain. Roher's observations have not been confirmed by others or by the author in the cases examined in the course of this study.

Merck's description of the gross appearance of the skin is sufficiently accurate, and no additions will be attempted. According to him, the essential features of the gross lesions are their limitation, usually, to the arms and the dorsa of the feet, early erythema or bullous formation followed by pigmentation, scaling, swelling, and wrinkling. By far the most common manifestation of the disease is the dry, pigmented, scaly skin.

Material and Method.—The material upon which these studies are based was procured from eleven cases autopsied in the Charity Hospital in New Orleans. The cases were diagnosed as pellagra by clinicians thoroughly accustomed to the different phases of the disease, and the autopsies were performed at periods of from two to eighteen hours after death. The tissues were removed, fixed in Zenker's fluid and 10 per cent. formaline, and were imbedded in paraffin. The sections were stained with eosin and methylene blue. For the determination of special tissues Mallory's connective tissue stain, Mallory's phosphotungstic acid hematoxylin stain, and Unna's orcein elastic tissue stain, were used. In the identification of pigment bodies, silver impregnation, after the manner of Levaditi, was resorted to.

Unfortunately there is little opportunity at autopsy to note the acute lesions. As a consequence no vesicles or bullæ were examined. The phenomena noted in several of the earlier cases have explained, however, the probable cause of these formations. In two cases the rash was erythematous in character; the other cases have shown a more or less marked pigmentation and scaling; in one case a brownish-black, dry, thickened mass of integument, measuring from 1 to 2.5 mm. in thickness was present over the dorsum of both feet.

Since the lesions in all the tissues that were examined are similar, a detailed description is given only of those cases which demonstrate the essential changes.

Case 1.—The patient was a white man, thirty-five years of age. At death the skin lesions consisted of a dry, pigmented scaliness over the dorsum of both feet, over the back of the hands, and below the olecranon of each arm. About the periphery of the darkened area over the forearm, a narrow zone of redness was present. Portions of the skin from the forearm and from the dorsum of the foot were studied.

The microscopical sections consisted of epithelium, corium, sebaceous and sudoriferous glands, etc., with a small amount of subcutaneous fatty tissue. The corium was normal in thickness and the skin appendages appeared negative. The superficial portion of the corium was very irregular in appearance, the normal papilliform arrangement being largely absent; the whole surface was, however, thrown into deep folds and projections. Over the outer surface of this papillary layer the epithelium was arranged. The deeper cells of the epidermis varied very much in character and followed the irregular outline of the fibrous tissue. In the *flatter* areas the columnar cells were shorter than usual in their long diameter. The prickle cell zone was composed of only one layer, and the stratum granulosum of but one or two layers of cells. In the *indented* portions the columnar cells were chiefly spindle-shaped, morphological differentiation between these and the underlying fibrous tissue cells being almost absent. Spindle-shaped columnar cells formed the greater part of the cellular thickness of the epidermis. The more superficial cells of this shape were prickled, there being no distinct polyhedral-shaped prickle layer. In the spindle columnar cells brownish-green pigment was present in the form of very minute granules; but it was practically absent in the cells covering the flatter portions of corium. There was a slight increase in the number of so-called Langerhans

cells (cells with long projections between and about the basal epithelial cells). Over the whole section there was a marked hyperkeratosis, the line of demarcation between the narrow zona granulosum and the keratinized layer being very clearly marked. The superficial layer of horny material is stained deep purple and appeared to be composed of four distinct layers. Beneath this purple-stained layer was a zone of about equal thickness consisting of pink-staining vacuolated bodies which had the general outline of cell bodies but were devoid of nucleus or other evidence of cell life. Throughout this area were numerous small, round and oval hyaline bodies similar to those described by Russell (8) in degenerating epithelium.

A similar hyperkeratosis was present throughout the walls of the hair follicles. It was especially marked beneath the skin surface. Diffusely throughout the corium, the tissues were separated by clear areas of edema.

In the superficial layers of the corium the blood vessels were increased in number and dilated. In the perivascular spaces collections of lymphoid cells were present. There were also a few lymphoid cells throughout the superficial layers of fibrous tissue. The papillary layer contained an unusually large number of cells, the nuclei of many of the fibrous tissue cells were large, and the protoplasm was irregular. Immediately beneath the epithelium were several cells whose morphology was equally suggestive of fibrous tissue and epithelial cells. These cells had fibers entering the deeper epithelial layers and contained no pigment. The sweat glands and nerves appeared normal.

Case 2.—The skin was from the dorsum of the foot of a negro man, thirty years of age. At autopsy, over both forearms and hands, but more especially over the dorsum of the feet, there was a marked thickening which was dry, scaly, and almost black in color. The sections consisted of epidermis, corium, and a small amount of subcutaneous tissue.

The epidermis was slightly thickened. It was divided distinctly into two layers. The outer layer, which was somewhat thicker than the inner, was composed of keratinized material; the inner layer was wrinkled, the distance between the indentations of the wrinkles measuring from .2 to 1 mm. There was no regular arrangement of the underlying corium, some places being flatter than usual, others showing deep projections of the epithelium. The cells of the Malphigian layer were normal in appearance and deeply pigmented, with the exception of one or two areas in which epithelial cells were placed below the regular epithelial border. The cells of the stratum granulosum were poorly differentiated from one another. The prickles on the cells in the deeper zones were very distinct. No mitotic figures were seen.

The upper layer of the corium was much rarefied and the collagenous fibrils were fine and sparse. There was an increase in the number of cells in this area, many of which resembled fibroblasts. Numerous pigmented, spindle-shaped, and branched cells were present. The smaller blood vessels were much dilated, and in the neighborhood of many of them numerous lymphoid cells were seen. The deeper layers of the corium were dense, the collagenous fibrils forming large masses, some of which were cloudy in appearance. Several of the larger vessels in the corium and in the subcutaneous tissue showed a separation of the muscle cells caused apparently by edematous fibrous tissue. In the hair follicles a marked hyperkeratosis was present. The musculares pili were swollen. The

sudoriferous glands were present, but were degenerated, as was evident from the presence of vacuoles and fine reddish granules in the epithelial cells. The nerves were apparently normal.

Case 3.—This patient presented the most aggravated form of the chronic lesion. Over the anterior aspect of both feet were large irregular areas of thick, dry, brown-black scaly eruptions. Similar eruptions, though less marked, were seen over the anterior aspect of both forearms just below the elbow and also over the dorsum of the hand, being especially marked in the triangular space between the metacarpal bone of the index finger and thumb.

Sections.—The skin from the dorsum of the foot consisted of epithelium and corium, together with a small amount of subcutaneous areolar tissue. The epidermis measured 2 mm. in thickness. With the low power of the microscope it was seen to be divided into two distinct layers, the superficial layer being composed of degenerated keratinized cells devoid of nuclei and taking a pale purplish stain.

Throughout the entire length of the section the layer of epithelium lying upon the corium was distinctly differentiated from the keratinized layer. The basal layer of epithelium dips down to a marked extent into the hollows between the papillæ of the corium. In places the individual basal epithelial cells projected irregularly into the rarefied corium. The cells forming the Malpighian layer were apparently normal. No mitotic figures were seen. In general the cells of the prickle layer were normal and the prickles were distinct. Over the papillæ were seen but one layer of prickle cells and but two layers of the stratum granulosum. Between the cells of the stratum granulosum and throughout the keratinized layer were numerous hyaline red staining bodies. Over the prolongations downward, the number of prickle cells was increased and the cells of the stratum granulosum consisted of three to four layers. The cells of the Malpighian layer contained a large amount of pigment. The cells of Langerhans were increased in number, their prolongations also being apparently somewhat longer than usual. These cells were especially numerous between the columnar cells and immediately beneath the epidermis, where they were identical in appearance with the chromatophores in the corium. A moderate amount of pigment was seen throughout the entire epithelial structure.

The whole corium was edematous. The superficial layers contained a markedly increased number of fibrous tissue cells; the collagenous fibers were decreased in number and very fine. There were also numerous oval cells with a deep red-staining protoplasm and a somewhat bean-shaped vesicular nucleus (young connective tissue cells). Numerous chromatophores were also present, especially beneath the epithelium. Many of the oval pigmented cells contained but one or two moderate-sized, brownish pigment granules; others which were much longer and narrower were completely filled with a similar pigment. In many places the prolongations of these cells projected in between the basal epithelium. The blood vessels in the superficial parts of the corium were numerous and dilated. About the blood vessels were moderate numbers of lymphoid cells. The lymphatic spaces were markedly dilated. No plasma or mast cells were seen. In that part of the corium which showed rarefaction of the collagenous fibers, there was a complete absence of elastic tissue fibrils. Deeper down were a few very fine fibrils, and in the deeper layers, which appeared normal, the

usual arrangement of elastic tissue was seen. One blood vessel, situated in the subcutaneous tissue, had a diameter of 1.5 mm. and showed a markedly thickened wall. The thickening was due, in part, to an increase in the medial layer, which was hyaline in appearance and contained few cells, but more especially to a proliferation of the endothelial lining with degeneration of the cells nearest the internal elastic lamina. The internal elastic lamina was broken up and much twisted. Numerous smaller blood vessels in the subcutaneous tissue showed a similar though less marked change. The epithelium of the sweat glands had degenerated, as was evidenced by vacuolization and the presence of round hyaline-staining bodies. No hair follicles were seen in the sections examined. The nerves appeared normal.

An area on the forearm showing a slight scaliness was also studied. The section of this consisted of epithelium and corium. The corium was similar to that in the last section, with the exception that the increase in the number of cells in the superficial layer was less marked. The elastic tissue fibrils in the superficial layers of the corium were swollen and apparently increased in number. This increase is, however, relative, and the result of the change in the intervening collagenous fibers. The sweat glands were normal. The epithelial layer was extremely narrow. The keratinized layer was normal or slightly increased in thickness. In general, the normal papilliform arrangement was absent, although there was some wrinkling over the greater part of the section. The prickle cells constituted but one layer, and the spindle cells of the stratum granulosum formed only one or two layers. The Malpighian layer was normal.

Another section from a part of the forearm, showing moderately well-marked hyperkeratosis, presented an appearance similar to that noted in the section from the dorsum of the foot, although the increased thickness of the epithelial layers was less marked.

The edema present in all these sections cannot be the result of local changes, since all the tissues of the body were water-logged, caused, apparently, by a general degenerative change in the smaller vessels. The alterations seen in the vessels in the subcutaneous tissue can, therefore, hardly be attributed to the local process, since the whole body presented a more or less marked general vascular change.

The lesions here are to be traced to a primary degenerative change in the corium followed by a reactionary process. The first change consists in the degeneration of the collagenous fibers with consequent rarefaction of the papillary layer of the corium and dipping down of the epithelial covering in search of a firmer base. The author questions that the changes in the blood vessels (*e. g.*, the dilatation of those in the superficial layers of the corium) cause the degeneration in the connective tissue, as has been suggested. To

him it seems more probable that the capillaries dilate as a result of the loss of support consequent upon the rarefaction of the surrounding tissue. Certain it is that the formation of new vessels is an active process and part of the reparative change. As a result of this increase in vascularity, the epithelial cells proliferate with the formation of the hyperkeratosis, which is characteristic of the lesions.

Although somewhat retarded, the changes in the elastic tissue are similar to those in the fibrous tissue. There is, in the earlier lesion, a swelling of the elastic tissue, with some fragmentation, and this is followed by complete destruction, as is evidenced by the absence of elastic tissue in the more advanced lesions. Replacement of the tissue apparently begins in the deeper zones.

Following the degeneration there is an attempt at repair, as is shown by the increase in the number of connective tissue cells and the presence of fibroblasts. The fact that such a reparative process took place, although the disease itself progressed, proves that the essential factor causing the degeneration comes from without and that it is not the result of toxic bodies in the blood stream. The presence, too, of an enormous increase in the pigmentation of the epithelium and the large number of chromatophores suggests that this irritant is sunlight or, at least, the ultra-violet rays, since we know that the production of pigment is the attempt of nature to protect the underlying tissues.

Case 4.—A section from the skin of the forearm, showing moderate pigmentation and scaliness, was impregnated with silver, reduction of silver taking place only in certain tissues in the upper layer of the corium and in the epithelium. The cells of the rete Malpighii were almost completely filled with black granules corresponding in size to the pigment granules noted with simple stains, but the black material in these sections was more abundant than the pigment seen in the sections stained by eosin and methylene blue. The granules were arranged chiefly about the periphery of the cell, a clear zone surrounding the nucleus. Owing to the extreme clearness of the sections, the protrusions of the basal epithelial cells into the corium were very distinctly seen, some of the fine prolongations projecting twelve microns or more into the connective tissue layer. The cells of the prickle layer and stratum granulosum also contained a large number of fine black granules. In general, the cells of the Malpighian layer contained the most silver, the stratum granulosum and stratum lucidum almost as much, and the prickle cells the least. The branching cells of the epidermis were most distinctly demonstrated and filled with silver deposit. A

definite net-work formed by the projection of these cells was present in the deepest layers.

In addition to the epithelial cells, there were seen, throughout the papillary layer of the corium, numerous cells of irregular shapes which were more or less filled with black granules. These cells were chiefly irregularly spindle-shaped, some were circular, and others were oval. In form these black cells resembled closely the chromatophores found in the sections stained with eosin and methylene blue; there were, however, a larger number present in this section than in the sections above described. It is noted, also, that, although the granules in many of these cells were very dark, they were slightly more brownish in color than the intense black granules in the epithelium.

Sections from two portions of skin from the first patient (p. 101) were similar to the one just described. In neither of these, however, was the gross lesion so marked and in neither was there such a large number of black cells in the corium, although both contained many cells with black granules. In the lesion showing least hyperkeratosis and least subepithelial rarefaction of the connective tissue, the number of cells with granules was smaller than in the one in which these changes were most marked.

The irregularity of the outline of the basal epithelium upon the connective tissues and the number and length of the prolongations in all sections varied, apparently, directly with the rarefaction of the corium; that is, with the loss in connective tissue fibrils.

Tissues from other cases, sectioned and infiltrated with silver, all showed similar changes, the number of granulated cells in the epidermis varying in all cases according to the extent of the rarefaction.

Apparently, then, in sections impregnated with a soluble silver salt, which was subsequently reduced, the pigment granules in the epithelium were found to be filled with metallic silver. The chromatophores of the corium and branching cells of the epidermis also contained particles of the metal. Not only does the brownish pigment, which is seen by other methods, take the salt in a manner which permits its reduction, but other bodies in similar cells also present the same reaction. In the absence of a like appearance in

other tissues, it seems probable that by this means we have a method for demonstrating the pigment bodies before they have become brown as well as after they have lost their color, as in the keratinized layer. This idea is also borne out by the larger number of black cells in the corium, demonstrated by the silver method, than brown pigmented cells seen in sections stained by simple methods.

In 1908 Schrieber and Schneider (9), as a result of observations based upon the preparation of foetal eyes by means of the Levaditi method, came to the same conclusion with reference to the deposition of silver in a reducible form in cells which contained melanin and also in cells in which the pigment as such had not appeared. They stated that not only colored pigment bodies, but also the antecedent colorless material, as well as the end products of the destruction of melanin, were capable of demonstration by means of this method. My own observations appear to corroborate their findings and conclusions.

The increase in pigment is one of the most constant changes in all skin lesions of pellagra. It is marked in those lesions which show the most advanced gross and microscopic lesions as evidenced by the hyperkeratosis and rarefaction of the corium. The pigment, which is greenish-brown in color, does not contain iron in a demonstrable form, occurs in small granules, and is, apparently, the same in both epithelium and mesoblastic cells. Nowhere is found an appearance suggestive of hematogenous pigment.

Councilman and Magrath (10) and others consider that the formation of pigment in such lesions is primary in the cells of the corium and is thence transmitted to the epithelium. The view has also been brought forward that the chromatophore of the corium derives its pigment from the epithelial cells. This latter view, however, may be almost excluded, since in the frog and other lower forms of life, chromatophores similar in appearance to those in the corium and containing a pigment indistinguishable from that in the skin, are present in the heart and other internal organs which contain no epithelial tissue. In the retina, a tissue composed exclusively of epiblastic cells, the production of pigment is more marked than in any other tissue of the body, proving the potentiality of epithelial cells to form melanin. The author considers, therefore,

that both epithelial and mesoblastic cells produce an autochthonous pigment, and that there is no reason for believing that the pigment, after production in the latter, is transmitted to the epithelial cells. It would be hard to explain the manner in which pigment so far removed from the surface could be directly turned over to the epidermis. The fact that pigmented mesoblastic cells lie, in some places, in very close proximity to the epithelium is explained more readily by considering that they occupy this position for the reason that the nearer the pigment lies to the surface of the body, the greater will be the protection to the susceptible connective tissue, than by assuming that they are passing their prepared melanin on to cells which appear quite capable of producing pigment.

Ehrmann (11), as the result of a comprehensive study of pigment production in the lower forms of life as well as in human embryos, considers that all pigment found in the adult epithelium has been primarily produced in cells which he says are neither connective tissue cells nor epithelium, and which he calls "melanoblasts." These "melanoblasts," according to his ideas, include the chromatophores of the corium as well as the branched cells seen in the epidermal layer, namely, the so-called Langerhans cells. He states that they are present in the latter position as a result of wandering from their original situation between the epiblast and mesoblast. As there appears to be no means of proving the source of the Langerhans cells of the epidermis, in this paper their histogenesis will not be discussed: nevertheless, as there is apparently undoubted evidence of the ability of the epithelium to manufacture pigment, I see no reason for using the term melanoblast, this term suggesting the future transposition of the pigment.

The degeneration and the reaction of the superficial layers of the corium, as shown by the loss in collagenous fibrils and degeneration in the elastic tissue, together with an increased cellularity and infiltration of the corium with lymphoid and plasma cells, is evidence of the effect of some irritant acting upon these tissues. The presence of an enormous increase in the pigmentation of the epithelium and, in particular, in the appearance of pigment-bearing cells in the corium is at least suggestive that this irritant is light. The presence of lesions uniformly and primarily in areas which are uncov-

ered or which are insufficiently protected against the sunlight, violet, or ultra-violet rays, is also evidence in favor of the importance of light in the production of the degeneration which precedes the reactive changes.

Several observers (12) have been successful in inducing an acute onset of erythema or vesicles by exposing to sunlight patients who, although suffering from pellagra, did not show skin manifestations. The fact, as demonstrated by Fox (13) and others, that once the lesions have appeared, protection from light is not effective in the cure of the conditions is explained by the fact that, with the exception of the initial lesion, the histologic phenomena are those of reparation rather than progressive degeneration.

If it be accepted that the changes are the result of the action of sunlight, some special predisposing factor must be present rendering the tissues less resistant than normally. Either this change takes place in the connective tissue of the corium or it is the result of some physiological alteration in the epidermis whereby the subepidermal tissues are not sufficiently protected.

In the early lesions of pellagra characteristics are noted which are histologically similar to those in an erythema caused by exposure to excessive sunlight and examined after the healing process has begun. There is dilatation of the blood vessels and lymph spaces together with a moderate serous exudate. Babes's (5) finding of polymorphonuclear leukocytes in cases showing vesicles is easily understood in the light of our general knowledge of the reaction of the tissues to injuries which are sufficiently acute. The metachromatic bodies described by him are probably degenerated collagenous material, and this we should expect to be most marked in severe lesions. In the material studied in this series, these early changes must be, in part, inferred. At first the epidermis is atrophied, and the prickle cells and stratum granulosum are decreased in thickness and show a tendency toward early keratinization.

Following the primary degenerative changes there is a reactive process consisting of a dilatation of the blood vessels and hyperplasia of connective tissue cells. As the disease progresses, in the superficial layers of the corium an infiltration of the perivascular tissues with lymphoid cells is seen. An increased

activity on the part of the epithelium takes place, together with an augmentation in the production of pigment. Although the deeper layers of epithelium are found to be growing rapidly and show a tendency to dip down into the rarefied corium, the upper layers, especially the stratum granulosum, show early degenerative changes resulting in the hyperkeratosis. The nerves at no time showed evidence of degeneration or fibrous tissue proliferation.

As pointed out by Wolbach (14) in his studies on X-ray dermatitis, the changes in the epithelium are apparently secondary to those in the corium. The histological appearance of the lesions of pellagra are, in many respects, strikingly similar to those described as the result of the action of the Roentgen light. There is the same degeneration of the collagenous and elastic tissue fibrils with secondary rarefaction and increased cellularity. These changes are followed by dilatation of the blood vessels, atrophy of the sweat glands, and, finally, by hyperplasia and rapid keratinization of the epithelial structures. The analogy between these two lesions is so marked that we are justified in considering that the direct agents in their production are probably similar.

Councilman and Magrath (10), from their investigations on xeroderma pigmentosa, in which, again, changes were found, similar to those in pellagra, are of the opinion that xeroderma develops as the result of some congenital condition, in consequence of which children are especially susceptible to the action of some irritable agent which is responsible for the development of the lesions. They consider that in all probability this causitive agent is sunlight, especially the ultra-violet rays. They refer to the apparently undoubted relationship between the formation of pigment and the action of light. According to their observations, atrophy of the corium is an essential lesion of xeroderma pigmentosa and is primarily responsible for the subsequent hyperkeratosis and tumor formation.

It has been demonstrated by physiologists that if the sun's rays be examined spectroscopically, after having passed through the cornea or other colorless epithelial tissue, certain rays at the violet and ultra-violet end of the spectrum are cut off. It is also known that the violet and ultra-violet rays are harmful to animal tissues,

and that they are apparently particularly harmful to connective tissues. If, as a result of an altered metabolism in the epithelial covering of the body, the substance, as yet not isolated, which normally filters out these ultra-violet rays, is not formed, the underlying connective tissue will be subjected to the detrimental action of these rays.

In my opinion it is more reasonable to suppose that the degeneration in the corium in both pellagra and xeroderma pigmentosa, is due, not to predisposition on the part of these tissues to the action of the violet and ultra-violet rays, but to an altered physiological function on the part of the epithelial cells, causing these to allow the passage of rays injurious to the subepithelial tissues. In exposure to the action of the Roentgen light, on the other hand, the surface of the body is influenced by rays against which there is no normal resistance on the part of the epithelium, with the result that there is a destruction of the upper layers of the corium.

The series of phenomena leading to the dermatitis of pellagra are, then, primarily, alteration in metabolism of the epithelial cells, whereby their power of preventing the ultra-violet rays from reaching the connective tissue of the upper layers of the corium is destroyed; as a result, the subepidermal tissues are subject to the action of these rays and are injured. The resulting degenerative process is followed by a stage of reparation accompanied by an increased vascularity, in consequence of which the epithelium proliferates rapidly with the development of the hyperkeratosis which was noted clinically.

It may be, also, that a similar loss of resistance on the part of the mucosa of the intestines, analogous to that found in the skin, predisposes to the development of lesions with symptoms referable to the intestines. Dysentery, or, at any rate, severe diarrhœa, is commonly met with during the course of the disease, and frequently the evidences of an acute colitis are found at autopsy. As has been shown by observations in our laboratory, the dysentery bacillus was in many cases the cause of the intestinal disturbances so frequent in the course of pellagra. It is now believed that the acid producing dysentery bacillus is present in many individuals as a normal inhabitant of the intestinal tract. It is, moreover, reasonable to

infer that, as the result of any circumstance leading to a lessened local resistance of the parts, autoinoculation takes place and the organisms develop pathogenic properties. This supposition is borne out by common experience. Other organisms, also, such as the colon bacillus and streptococcus, are recognized as being potent to induce, under certain conditions, catarrhal and other inflammations of the intestines. It seems unreasonable to suppose that the essential factor in the production of pellagra, whatever this may be, should produce a lessened resistance to one irritating body only. Up to the present, no histological changes in the intestines have been described to account for the occurrence of the diarrhœas, and although it is possible that a functional insufficiency on the part of the nerve plexuses may be the cause, this explanation is not very satisfactory. On the other hand, there is cause to believe that, whereas, in the case of the epidermal covering of the body, this loss of resistance is against the ordinary irritating agent to which it is accustomed, namely, sunlight, the loss of resistance upon the part of the entodermal lining may, in a similar manner, be against an irritant to which it is normally subjected, namely, the bacterial flora of the intestinal tract and their toxines. In a similar way functional changes in the epithelial cells of the mucosa in the mouth and genitals would cause the lesions described in these situations. If this theory be accepted, the occurrence of at least two important evidences of the disease can be explained, and also the lack of either or both in any individual case.

Pellagra is a disease characterized by certain organic changes in the skin and intestines, but more especially by certain nervous manifestations, for which, up to the present, no constant histological factors have been recognized. If the changes in the skin be explained primarily by an altered function on the part of the epithelium, may it not be that the symptoms referable to the nervous system are the result of an altered metabolic activity of the nerve cells, which are also epithelial in origin? Such an explanation, in the absence of histological alterations in the nervous system, seems tenable.

It is my belief that pellagra is a disease in which, under the influence of some toxic substance or microörganism, there is an altered function on the part of the epithelial tissues throughout the

body, rendering them less resistant than normally to the action of different injurious agents. Whether the altered function on the part of the epithelium be the result of the toxic action produced by low forms of vegetable life outside the body or the action of some unknown microörganism or protozoon in the body, need not be discussed in this paper.

SUMMARY.

The cutaneous lesions in pellagra consist of an early erythema, or, in occasional cases, of vesicles or bullous formations which are followed by hyperkeratosis and pigmentation, resulting in a dry, dark brown scaliness. These various lesions are similar to those normally produced by the action of sunlight, but are much more marked. The histological phenomena of the erythematous and bullous stage are those of a mild acute inflammatory reaction, together with a degeneration in the superficial layers of the corium.

Following this degeneration, which involves not only the general connective tissue but the connective tissue of the blood vessels, there is a reparative change evidenced histologically by an increased cellularity of the corium and the presence of fibroblasts. The capillaries also are increased in number and much dilated. Apparently as a result of this increased vascularity of the corium, there is an increased proliferation of the epithelium resulting in a thickening of the epidermis. This increase in thickness of the epithelial layer is especially marked in the prickle cells and the stratum granulosum. In the later stages, in an effort to secure a firm basement membrane, the epithelium is seen to dip down deeply into the rarefied connective tissue. About the blood vessels during the reactionary process are found collections of lymphoid cells, a few plasma cells, but no mast cells or eosinophiles.

That the irritant producing the degeneration in the corium is sunlight in the presence of some predisposing factor, is suggested by the enormous increase in pigment formation in the epithelial cells and by the large number of chromatophores in the superficial layers of the corium. This pigmentation is autochthonous in both types of cell. There is no reason for believing that the pigment is formed in the cells of the corium and thence discharged into the epithelium, or that the reverse process takes place.

The predisposing factor inducing the changes in the corium is, apparently, a lessened resistance of the epithelium to the violet and ultra-violet rays, due to some metabolic insufficiency on the part of the epithelial cells.

Further observation may justify the conclusion that throughout the body, pellagra is a disease essentially of the epithelium, including the nervous system, this pathological condition manifesting itself by an insufficient or altered function.

In conclusion I wish to express my thanks to Dr. Duval and the clinicians of the Charity Hospital for many helpful suggestions.

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EXPLANATION OF PLATE V.

FIG. 1. Photograph of the hands of a pellagra patient who had worn rings. Those portions of the fingers protected from the sunlight are free from the skin lesions. I am indebted to Dr. C. C. Bass for the use of this photograph.

FIG. 2. Section of skin from case 3, showing hyperkeratosis and dipping down of the epithelium into the rarefied corium. Note the extreme dilatation of the vessels in the superficial layer of the corium.

FIG. 3. Specimen prepared by Levaditi's method, showing a large number of pigment cells in the corium.

FIG. 1.

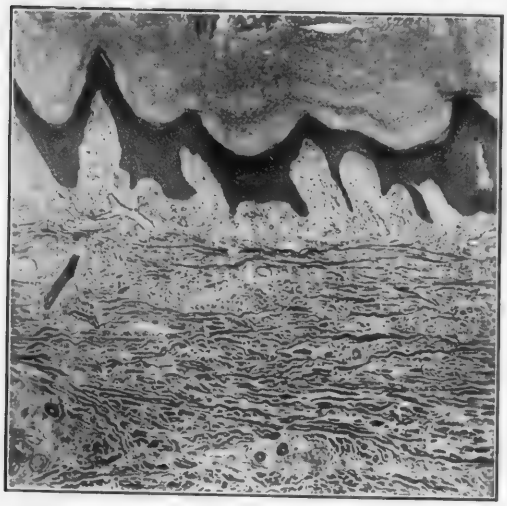

FIG. 2.

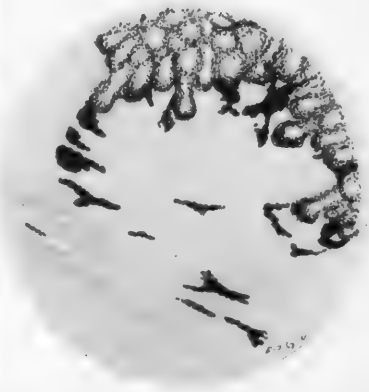

FIG. 3.

THE PATHOGENESIS OF ICTERUS.[1]

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This paper deals with the pathogenesis of obstructive jaundice, and perhaps throws some light on the mechanism of escape of the bile pigments from the bile capillaries and the appearance of the same pigments in the urine. If justification for this study is needed, it may be found in a review of the work done during the past few years, and in the widely varying results of different investigators. Statements in many of the text-books are at variance and a few quotations may be permitted in this place.

A. O. J. Kelly (1) states, "That the pathway of the bile to the blood vessels is by way of the lymphatics and the thoracic duct has been repeatedly demonstrated, . . . and if the common bile duct and the thoracic duct be ligated, biliary pigments do not appear in the urine for many days." Rolleston (2) takes substantially the same view.

Adami (3) says, "Clearly, the normal path by which the bile reaches the circulation is by way of the lymphatics." And further, "It also has been clearly demonstrated by Vaughan Harley (4) . . . that if two dogs be taken, and in both dogs the common bile duct be ligated, and in one, in addition, the thoracic duct be also closed, in the one bile pigment appears in the urine (discharged from the blood) in the course of a few hours; in the other, with the thoracic duct closed, it may be eight or even fourteen days before there is any such discharge."

Heidenhain (5) states that, after ligation of the common bile duct, the lymph in the thoracic duct contains large amounts of bile pigments and salts, but the blood serum does not contain a trace of either. He concludes, therefore, that the bile must escape into the perivascular lymphatics, from there to the

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larger lymphatics, and finally into the blood through the thoracic duct. Gerhardt (6), in several communications, confirms the findings of Heidenhain.

Eppinger (7) has contributed several excellent papers on this subject and, in his last paper, gives a careful review of the extensive literature. His views are based on a very careful study of histological preparations in which the bile canaliculi are specially stained. From the study of this material he cannot accept the theory of rupture of bile capillaries and escape of bile into the hepatic capillaries, but is convinced that after rupture of the bile capillaries the bile escapes into the perivascular lymphatics. The bile is drained into the thoracic duct by the larger hepatic lymphatics. He believes the same explanation holds for practically all types of icterus, obstructive, toxic, etc.

Minkowski (8) offers an interesting explanation which is opposed to the theories above stated. He remarks that it is well known that the liver cells can secrete substances into the hepatic capillaries, for example, uric acid and sugar. Why cannot the liver cells under abnormal conditions secrete bile into these hepatic capillaries? He believes that mechanical stagnation of the bile leads to damage of the liver cells and disturbance of their secretory function which may bring about a *reversal* of the secretion. He compares this change with that produced in the renal epithelium allowing an escape of albumen in the urine.

Nauwerck (9) is a strong advocate for absorption of bile in obstructive jaundice by way of the hepatic capillaries, and bases his views on a careful microscopical study. He states that the bile capillaries communicate with the blood capillaries.

Starling and Tubby (10) injected indigocarmine and methyl blue into the pleural and peritoneal cavities of animals and noted that the color first appeared in the urine long before its appearance in the fluid of the thoracic duct. They drew the conclusions that absorption by blood vessels was not only far better, but they questioned whether any took place by way of the lymph, as the moderate coloration might have come in later from the blood, after the coloring matter had been directly absorbed by the blood.

Meltzer (11) repeated these experiments, with some modifications, and found that the color appeared in the lymph before it did in the urine.

Wertheimer and Lepage (12) bring strong experimental evidence to show that the lymphatics play a subsidiary rôle in the absorption of bile, which goes on actively by way of the hepatic capillaries and is in no way influenced by a thoracic duct fistula. They inserted canulæ in the thoracic duct, the common bile duct, and the urinary bladder. Injection of a solution of sodium indigo sulphate or bilirubin into the bile ducts under a pressure of 30 to 35 cm. caused these substances to appear in the urine by the end of two hours.

Mendel and Underhill (13) repeated and amplified these experiments of Wertheimer and Lepage, with similar and even more conclusive results. They used a solution of potassium iodide which was introduced into the bile ducts and appeared in the urine at the end of twenty to thirty minutes. A little later the drug appeared in the lymph of the thoracic duct, but always in less amount than in the urine. They concluded that the hepatic capillaries are the important factors in this absorption. Mendel (14) has shown that in absorption from the peritoneal cavity the pigments appeared in the urine before they did

in the lymph, even when a lymphagogue had been previously given to insure an abundant flow. The tardy appearance of pigment in the lymph cannot be explained as due entirely to a retarded flow, but is due, in part at least, to a variable pressure of bile in the ducts and canaliculi.

A recent contribution by Herring and Simpson (15) has interested us considerably and we quote from their summary. "The obstructed bile leaves the liver by the portal lymphatics. Injection of the bile ducts with carmine gelatin at a pressure not exceeding the maximum bile pressure is also followed by the appearance of the injected material in the liver lymphatics." Obvious objections may be raised to this type of injection experiment. A careful review of their experiments does not convince one that many sources of error have been excluded. The method of testing for bile in the portal lymphatics is quite inaccurate; nor are we convinced that such experiments offer sufficient evidence for their assertion that "the obstructed bile leaves the liver by the portal lymphatics."

It may be of interest here to recall the anatomy of the liver lobule with especial reference to the lymphatic system. Mall (16) states that the lymphatics are present only in the centers of the portal units and are absent in the centers of the liver lobules. Presumably they drain the tissue intimately associated with Glisson's capsule and the portal structures.

To summarize in a few words, many investigators explain the escape of bile from the liver in obstructive jaundice by the activity of the *lymphatic apparatus,* while others uphold the view that this process is essentially an absorption of bile from the liver by the activity of the *hepatic capillaries.*

It was with the idea of clearing up this question that our series of experiments was undertaken. We have tried to simulate, as closely as possible, the conditions found in acute obstructive jaundice, have recorded our observations upon the lymph and urine, and then permitted the animal to live for some time. After an interval of days or weeks the animal was examined for the presence of bile pigments in the various body fluids, and this throws some light on the pathogenesis of chronic jaundice. It is believed that such experiments, extending over a long period of time, are more valuable than shorter ones in which no aseptic precautions were taken, and in which the animal was sacrificed at the end of a few hours.

Method.—Large female dogs were used. The urine was obtained by catheter, and in every case was examined before operation.

It was found that the only sure method of producing permanent obstructive jaundice in dogs was to ligate the common bile duct doubly and to cut it

between the ligatures. A single ligature cuts through the duct, and its continuity is reëstablished in a few days. Ether was used for anesthesia, no morphia being given as, in some of the early experiments, this seemed to interfere with the excretion of bile. Unless otherwise stated, the Salkowski test was employed for bile pigments, and the Hoppe-Seyler test, for bile salts. Whenever the material to be examined was scanty, we used the Salkowski test for bile pigment, as it is much more delicate than that for bile salts (Hoppe-Seyler), and in our experiments in every instance in which the presence of bile salts has been demonstrated, the bile pigment test has been more marked.

We shall report in some detail a few of our more complete experiments, but the findings in all are in accord. Although in some details a few experiments are incomplete, the weight of evidence points to the conclusion that in obstructive jaundice bile is absorbed from the liver chiefly by way of the hepatic capillaries and that the lymphatics play a secondary rôle.

Dog 102.

April 21. The dog was a large mongrel pointer and weighed 43¼ pounds. The animal ingested about one pound of fat meat and one pint of milk at 8.45 a. m. The urine obtained at this time, by catheter, gave a negative bile pigment test. Ether anesthesia was begun at 10.25 a. m. The *thoracic duct* was exposed at the root of the neck and tied at its junction with the jugular vein. A canula was inserted distally to this ligature, and a uniform flow of lymph was obtained at 11.50 a. m. The lymph was rather milky and the flow constant, between 2 and 5 c.c. per fifteen minutes.

A second operation was performed at once through the right rectus. The *common bile duct* was ligated doubly and cut between ligatures at 12.45 p. m. Saline infusion was given continuously during both operations, 1,500 c.c. of salt solution (0.8 per cent.) being introduced subcutaneously during the three hours in which the animal was under the anesthetic. In spite of this, the flow of urine was rather scanty but regular, and the urine was highly colored. The animal was permitted to come out of the anesthesia at 1.30 p. m., but urine and lymph were collected regularly during the next four hours. When the animal was placed in a metabolism cage at 6 p. m., the lymph was still flowing regularly. The tests of the urine and lymph follow.

April 22. At 10 a. m. the dog was well and lymph was still flowing from the thoracic fistula, about 100 c.c. having collected during the night. There were large clots of lymph in the cage and collecting vessels. Ten cubic centimeters of clear amber-colored lymph were collected from the fistula and gave a bile pigment test. Urine (catheterized) was not highly colored, but gave a positive bile pigment test. At 3 p. m. the lymph flow was still regular and abundant, about 3 c.c. every five minutes. The lymph had a clear, amber color and 4 c.c. of it gave a suspicious test for bile pigment. The urine obtained at this time by catheter gave a very beautiful bile pigment test.

Urine			Lymph.		
Time.	Amount.	Bile pigment.	Time.	Amount.	Bile pigment.
Before operation.	10 c.c.	Negative.	Before operation.	—	—
April 21 12.45 p.m.	Common bile duct ligated.				
1.10 p.m.	12 c.c.	Negative.	1.30 p.m.	2½ c.c.	Negative.
1.30 p.m.	3 c.c.	Negative.	1.45 p.m.	4½ c.c.	
1.50 p.m.	5 c.c.		2.00 p.m.	5½ c.c.	
2.05 p.m.	2½ c.c.	+	2.15 p.m.	4½ c.c.	Negative.
2.20 p.m.	3 c.c.		2.30 p.m.	2½ c.c.	
2.40 p.m.	3½ c.c.		2.45 p.m.	3 c.c.	
3.00 p.m.	2 c.c.	++	3.00 p.m.	2½ c.c.	Negative.
3.40 p.m.	4.3 c.c.		3.15 p.m.	2 c.c.	
4.00 p.m.	2.7 c.c.		3.30 p.m.	1½ c.c.	
			3.45 p.m.	3 c.c.	
4.15 p.m.	3½ c.c.	++	4.00 p.m.	1½ c.c.	
4.30 p.m.	5 c.c.		4.15 p.m.	1½ c.c.	
5.00 p.m.	2½ c.c.		4.30 p.m.	1 c.c.	
			4.45 p.m.	2 c.c.	Negative.
5.30 p.m.	6 c.c.	++	5.00 p.m.	2 c.c.	
			5.15 p.m.	2 c.c.	
			5.30 p.m.	1½ c.c.	
April 22 10 a.m.	8 c.c.	++	10.00 p.m.	10 c.c.	++
3 p.m.	10 c.c.	+++	3.00 p.m.	4 c.c.	Negative (?)
April 23 5 p.m.	5 c.c.	++		No lymph.	
April 24.	5 c.c.	++		No lymph.	

April 23. The dog was pretty well; a little lymph was flowing from the neck wound. The urine was clear orange yellow and gave a positive bile pigment test.

April 24. The dog was improving and the wounds were granulating well. The urine was typical of obstructive jaundice, and a catheterized specimen gave a deep orange yellow color with a green froth.

April 28. The dog was quiet and its abdomen seemed tender. The urine was very highly colored and typical of obstructive jaundice.

May 5. The dog was sick and weak and was killed with ether.

Autopsy.—This was performed at once. All the subcutaneous tissues showed a deep bile tinge. The serous cavities were clear except for old adhesions around the site of operation on the common bile duct. The heart was pale. There were a few subendocardial ecchymoses. The lungs were crepitant, as a rule, but showed scattered small patches of deep purplish color. These measured 4 or 5 mm. in diameter, stood up above the cut section, and were evidently areas of consolidation. The spleen was large and soft. The cut section was velvety. The stomach and duodenum were quite normal. The parenchyma of the pancreas was rather pale and bile-tinged. All through the parenchyma, particularly in the head of the pancreas, were scattered many, small, chalky opacities, about pin-head in size.

Liver.—This was bile-tinged but of a deep purplish color due evidently to intense congestion. The common bile duct and gall bladder were enormously swollen and very tense. The kidneys presented a remarkable appearance. They were large and the capsule came off easily, leaving a mottled surface on which areas of bile-stained cortex alternated with purplish areas stippled over with little yellow specks. Sections showed a cortex striated in a similar manner. Areas of hemorrhage were streaked with opaque, yellow colored lines which extended downward through the pyramids to the pelvis. The pelvis was dilated and full of purulent material. The tips of the pyramids showed some ulceration and erosion and had a rough, shaggy, dirty yellow appearance. The ureters contained a purulent material and showed injection of their mucosa. The bladder contained some gas which escaped on section. The mucosa was everywhere hemorrhagic. There were some raised areas around the trigonum in which one could see tiny blebs which were filled with gas and measured 1 to 3 mm. in diameter. These little bubbles were present everywhere in the mucosa, gave a honey-combed appearance to these areas, and were undoubtedly due to a gas bacillus infection. This interesting condition had no bearing on our problem; it probably developed from an infected bladder which caused an ascending pyelonephritis and terminal bronchopneumonia.

Dog 112.

July 20. The dog was a great Dane, apparently of pure breed, and weighed 72 pounds. She was rather old and lean but was active and powerful. The animal was isolated for observation. She ate vigorously and appeared normal in every respect.

July 21. The animal was in perfect health, and the urine obtained by catheter was negative for bile pigments.

July 23. At 7.30 a. m. the animal ingested one pound of chopped fat meat and one quart of milk. These were taken at once. The animal also drank considerable water. The operation under ether anesthesia was begun at 10.20 a. m. A subcutaneous saline infusion was given continuously from 10.30 to 1 o'clock. The saline infusion (0.8 per cent.) was absorbed rapidly and 2,000 c.c. were given in all. Thiosine (.04 gram) was given subcutaneously at 10.45. The thoracic duct was exposed and ligated at its entrance into the jugular vein, and at 11.15 a large canula was easily inserted into it and tied. A copious flow of milky, opaque lymph was obtained. This averaged about 1 c.c. per minute, as is indicated in the table. In the second operation under the same anesthetic an incision was made through the right rectus, and the common bile duct was doubly ligated and cut at 11.45. Five grains of caffein were given in the saline infusion at 12 m. and again at 1 p. m. Ether was administered continuously between 10.20 a. m. and 3.30 p. m. (1,800 c.c. in all). Observations, with regular collections of urine and lymph, were continued until 4.45 p. m. During the last hour the lymph was pretty clear and very slightly tinged with blood. The animal stood the operation well and recovered sufficiently to walk before it was returned to its cage. It vomited several times that night.

The results of the urine and lymph tests are given in the following table.

	Urine.		Lymph.	
Time.	Amount.	Bile pigment.	Amount.	Bile pigment.
July 21, 4 p.m.	20 c.c.	Negative	——	——
July 23, 10.30 a.m.	20 c.c.	Negative		
11.40 a.m.	8 c.c.	Negative	20 c.c.	Negative
11.45 a.m.	Common bile duct ligated.			
12.00 p.m.	3½ c.c.	++	14½ c.c.	+
12.15 p.m.	3½ c.c.		13 c.c.	
12.30 p.m.	2 c.c.		12 c.c.	
12.45 p.m.	3 c.c.		12½ c.c.	
1.00 p.m.	3½ c.c.	++	21 c.c.	Faint +
1.15 p.m.	2 c.c.		12 c.c.	
1.30 p.m.	2 c.c.		13 c.c.	
1.45 p.m.	3 c.c.		12 c.c.	
2.00 p.m.	3 c.c.	+	13½ c.c.	Negative
2.15 p.m.	2½ c.c.		12½ c.c.	
2.30 p.m.	2 c.c.		19 c.c.	
2.45 p.m.	1 c.c.		14½ c.c.	
3.00 p.m.	2½ c.c.		7 c.c.	
3.15 p.m.	2 c.c.		13 c.c.	
3.30 p.m.	3½ c.c.	+	12½ c.c.	++
3.45 p.m.	4 c.c.		14 c.c.	
4.00 p.m.	4 c.c.		15 c.c.	
4.15 p.m.	3 c.c.		11 c.c.	
4.30 p.m.	2 c.c.		13 c.c.	
4.45 p.m.	3½ c.c.		21½ c.c.	
July 24	10 c.c.	+++	——	No lymph obtained.
July 25, Autopsy	2 c.c.	++++	1½ c.c.	+ (Faint)

July 24. At 12 m. the animal seemed pretty well and drank a large amount of water. No food was given. She walked about the room and appeared fairly normal except for considerable weakness and a slight increase in the rapidity of respiration. Urine was obtained by catheter in considerable amounts. It was very highly colored and appeared definitely bile-tinged.

July 25. At 9 a. m. the animal was found dead and rigid. At autopsy, jaundice was evident in the sclerotics, and there was a faint tingeing of the subcutaneous tissues as well. The abdominal wound was clean and the tissues about it were rather edematous and injected. The peritoneal cavity contained 50 to 70 c.c. of turbid, dirty, brownish-yellow fluid which evidently contained a good deal of bile. The serous surfaces were all injected, particularly in the neighborhood of the liver. The pelvis was fairly clear. The duodenum was greatly dilated, and its walls were injected. There was a conspicuous fibrino-purulent exudate appearing in patches on the surface of the liver. There was a large mass of this material close to the gall bladder in one of the small fissures between two of the liver lobes. There was another accumulation of fibrin

deep down in one of the liver fissures close to a larger bile duct. Careful examination showed no gross evidence of rupture, but it seemed pretty clear from the localized type of the exudate and the appearance of the liver after removal that there had been a small rupture, permitting the escape of more or less bile. This condition is not uncommon after ligation of the common bile duct in dogs, but usually occurs from the distal end of the gall bladder or from one of the larger bile ducts at the hilum of the liver. Chemical examination showed the peritoneal fluid to be very rich in bile pigment, whereas the pericardial fluid, which is a fairly good index of the general distribution of the bile pigments in the body fluids, contained only a very small amount. This was additional evidence in favor of an escape of bile pigment through a rupture in the liver. The pleural and pericardial cavities had moist, smooth surfaces. A small amount of fluid was obtained from the pericardial cavity.

Lungs.—The right lung was practically normal. On section the left lung was very large, heavy, and moist, and of a deep purple color, particularly in the lower lobe. There were purple patches in the other lobes. There was evidently a good deal of bronchopneumonia and edema affecting particularly the lower lobe. The spleen, pancreas, kidneys, duodenum, and stomach showed nothing of interest.

Liver.—This was large and swollen. The lobules were quite conspicuous, showing a swollen, grayish-red margin and a deep red center. The acute exudate on the capsule was described above. The gall bladder was dilated to about double its size and was quite tense. The common, cystic, and hepatic ducts were all dilated and conspicuous. The site of the operation showed that the ligatures were intact and that there had been no escape of bile from this region. The lymphatics at the hilum of the liver were dilated, beaded, and made out with ease. They were filled with a lemon yellow colored fluid. The urinary bladder contained about 50 c.c. of very highly colored urine, which was removed with a clean pipette and was found to contain an enormous amount of bile pigment. In consequence of the exudate in the peritoneal cavity, the thoracic duct contained a clear, pale, cherry-red fluid (compare dog 82), which was obtained through a canula.

At autopsy the urine, lymph, and ascitic and pericardial fluids were examined under similar conditions. The result is given in the following table.

	Urine.	Lymph.	Ascitic fluid.	Pericardial fluid.
Amount	2 c.c.	1½ c.c.	2 c.c.	1¾ c.c.
Color.	Orange	Cherry-red	Dirty brownish yellow	Pinkish amber
Bile pigment . .	++++	+	+++	+ (Faint)

It might be argued that the icterus in this case was due in great part to the peritonitis and bronchopneumonia which resulted fatally, but we believe that these were slight factors. The urine at autopsy was very rich in bile pigments and the course of development of the

icterus followed the same curve as in the other experiments. In dogs, hematogenous icterus resulting from peritonitis or bronchopneumonia is usually faint, bile pigment being present only in traces in the body fluids.

The experiment with dog 112 seems in part to contradict the first one with dog 102. We believe, however, that this contradiction is apparent only. In dog 102 the lymph flow and, presumably, the digestive activity were not particularly active. The bile pigments were either not present in the lymph, or were there in quantities insufficient to give the Salkowski test. In the urine, however, the bile pigments appeared during the second hour after the ligation of the common duct. The experiment with dog 112 was performed during the height of digestive activity and the lymph flow was very abundant, about five times as abundant as the flow of urine. Here, during the *first* hour after ligation of the common bile duct, both the urine and lymph contained bile pigments in demonstrable amounts. This would indicate a very rapid secretion and escape of bile from the canaliculi with removal of the bile by both the blood and the lymph channels. In spite of the relatively small amount of urine, the tests were as strongly positive in the urine as in the lymph, except during the last hour.

The experiments with dogs 102 and 112 show conclusively that a thoracic duct fistula influences in no way the development of icterus in dogs, and indicate that the lymphatic apparatus plays an unimportant and fluctuating rôle in the absorption of bile pigments from such a liver. In the first animal (dog 102) the lymph flow was not very abundant, and it is possible that a copious flow of lymph may sweep more of the bile into the thoracic duct. A more probable explanation is that it depends upon the rapidity of bile secretion in the liver. When bile is formed very rapidly it causes a rapid rise of pressure within the bile ducts and a rapid escape of bile from the canaliculi. Some of the bile may be absorbed and swept away by the lymphatics before it is absorbed by the blood vessels. It is possible that a small percentage of the bile pigments always does escape by way of the lymphatics, but only when the flow is sufficiently active is enough secreted to give a positive Salkowski test.

There is another possible and very simple explanation. In no

experiment of ours has bile pigment appeared in the lymph of the thoracic duct before it was demonstrable in the urine, and usually the bile pigments, if present in the lymph, were found there later than in the urine. It is obvious that these same pigments must be present in the serum before they appear in the urine, so it is quite possible that the pigments may be taken up by the lymphatics from the blood serum and tissues bathed in this serum.

This explanation applies to the liver and to all body tissues. The findings in the pericardial fluids, given below, support this view, for in chronic jaundice we find the same concentration of bile pigments in the pericardial fluid as in the fluid of the thoracic duct, and this concentration is, as a rule, less than that of the blood serum (dog 89). The anatomy of the liver lobule suggests the possibility that the lymph is drained only from those liver cells which are close to the portal tissue.

Dog 79.

December 17. This animal was a long-haired mongrel setter and weighed 30¾ lbs. The urine obtained by catheterization was negative for bile pigments and bile salts.

December 22. At 10 a. m. the animal was given one pint of milk which it drank immediately. The operation under ether anesthesia was begun at 11.30. The thoracic duct was exposed, tied at its junction with the internal jugular vein, and into it was inserted a small glass canula. A uniform flow of lymph was obtained, about 3 c.c. every fifteen minutes. The second operation was performed at once. The common bile duct was ligated doubly and cut at 1.30 p. m. Beginning at one o'clock, salt infusion of 500 c.c. was given subcutaneously for one hour. The flow of urine was about 2 c.c. every fifteen minutes.

December 23. At 11 a. m. the animal was quite well. The abdominal wound was rather edematous. The urine gave a strong positive test for bile pigments and bile salts.

December 24. The dog was well and had a good appetite. The urine had the typical appearance of obstructive jaundice.

December 25 and 26. The animal was bleeding slightly from the granulating operative wound in the neck.

December 30. The urine was very highly colored and its foam was greenish. The animal died as the result of a large hemorrhage from the vessels in the neck.

Autopsy.—The peritoneum was quite clean and free from bile. There were old adhesions about the site of operation. The gall bladder and bile ducts were enormously dilated and very tense. All the tissues showed a striking jaundice tinge. The fatal hemorrhage was due to rupture of one of the larger veins at the bottom of the operative incision at the root of the neck.

Urine.			Lymph.		
Time.	Bile pigment.	Bile salts.	Time.	Bile pigment.	Bile salts.
Before operation.	Negative.	Negative.	Before operation	Negative.	Negative.
Dec. 22, 1.33 p. m. Common bile duct ligated.					
1.55 p.m.	Negative.		1.55 p.m.		
2.05 p.m.			2.05 p.m.		
2.15 p.m.	+		2.15 p.m.	(?)	
2.30 p.m.			2.30 p.m.		
2.45 p.m.			2.45 p.m.		
3.00 p.m.			3.00 p.m.		
3.15 p.m.		+	3.15 p.m.		Negative.
3.30 p.m.			3.30 p.m.		
3.45 p.m.			3.45 p.m.		
4.00 p.m.	Negative (?)		4.00 p.m.	Negative.	
4.15 p.m.			4.15 p.m.		
4.30 p.m.			4.30 p.m.		
4.45 p.m.	+		4.45 p.m.		
5.00 p.m.			5.00 p.m.		
Dec. 23 11.00 a.m.	++	++	Dec. 23 11.00 a.m.	—	— Not enough lymph obtained.

Dog 86.

January 20. The dog was a large St. Bernard and weighed 60 pounds. At 9 a. m. the animal drank a quart of milk, and at 10 a. m. the operation was begun. The thoracic duct was isolated, tied at its junction with the jugular, and a glass canula was inserted. The lymph flow was abundant, as is indicated in the table. A subcutaneous saline infusion of 500 c.c. was given at 10 o'clock. Under the same anesthesia, the common bile duct was isolated close to the duodenum, doubly ligated, and cut at 1.50 p. m. There was some bleeding into the peritoneum from rupture of a small vessel close to the common bile duct, and this may explain the slight blood tinge in the lymph after the operation. The urine had a constant flow of about 4 to 6 c.c. every fifteen minutes. The lymph flow was also very constant, about 12 to 15 c.c. every fifteen minutes.

The results of the urine and lymph examinations are given in the table that follows.

January 22. The dog was sick and refused to eat. Some blood was lost from the wound in the neck. There was a bad diarrhea and the urine was contaminated with fecal matter. Repeated catheterizations yielded no urine.

January 24. The animal was weaker and refused to eat.

January 25. The animal was moribund at 11 o'clock. It was killed with ether and the autopsy was performed at once. Three hundred cubic centimeters of blood were obtained from the vena cava, defibrinated, and centrifugalized.

The *serum* obtained had a clear whiskey color and showed on shaking a greenish froth. It gave a strong test for bile pigments. The bladder contained

Urine.				Lymph.			
Time.	Amount.	Bile pigment.	Bile salts.	Time.	Amount.	Bile pigment.	Bile salts.
Before operation	—	Negative	Negative	Before operation	—	Negative	Negative
Jan. 20, 1.50 p.m. Common bile duct ligated.							
2.05 p.m.	5 c.c.						
2.20 p.m.	3½ c.c.						
2.35 p.m.	4 c.c.						
2.50 p.m.	4 c.c.		Negative	2.50 p.m.	19 c.c.		
3.05 p.m.	6 c.c.	Negative		3.05 p.m.	20 c.c.		
3.20 p.m.	4½ c.c.			3.20 p.m.	24 c.c.		
3.35 p.m.	7 c.c.			3.35 p.m.	7½ c.c.		
3.50 p.m.	8 c.c.	?		3.50 p.m.	11 c.c.		Negative.
4.05 p.m.	4½ c.c.			4.05 p.m.	7½ c.c.	+Faint	
4.20 p.m.	6½ c.c.	+		4.20 p.m.	2½ c.c.		(78 c.c. in amount)
4.35 p.m.	7 c.c.				—		
4.55 p.m.	8 c.c.	Faint +			No flow		
5.25[1] p.m.	11 c.c.			5.25 p.m.	10 c.c.		
5.40 p.m.	13.5 c.c.	+		5.40 p.m.	19 c.c.	+	
5.50 p.m.	7 c.c.			5.50 p.m.	5 c.c.		
Jan. 25	2 c.c.	+			5 c.c.	++	

2 c.c. of very turbid urine which gave a positive test for bile pigments. The bladder mucosa was swollen and pinkish, and the cystitis undoubtedly explained the frequent micturition which made it impossible to obtain urine on the days following the operation. The peritoneal cavity showed a fresh, fibrinous exudate over the liver and a little turbid fluid in the flanks. The serous surfaces were injected and slightly granular. The pleural and pericardial cavities were clear. The heart, lungs, spleen, pancreas, kidneys, and intestinal tract were normal in gross and in microscopical examination. The liver was enlarged and showed throughout a definite icteroid tint. Its capsule was covered with a slight fibrinous exudate. In addition, one could see everywhere beneath the capsule minute yellow specks. Microscopical sections showed these specks to be rather large, recent focal necroses. Many of the bile canaliculi were distended with little greenish casts. The gall bladder and larger bile ducts were greatly distended with very tenacious, black, slimy bile. The tissues everywhere, particularly the subcutaneous and subserous fat, showed a definite icteroid tint. The *thoracic duct* contained 5 c.c. of highly colored, slightly blood-tinged fluid, which was obtained through a clean glass canula. Bile pigment tests were strongly positive.

The postmortem findings in this animal (dog 86) were very interesting. The highly colored blood-tinged fluid in the thoracic duct is explained in part by the acute peritonitis and the focal necroses in the liver. It is interesting to compare this experiment with that of dog 82 (p. 127) in which there was a peritonitis due to rupture of the gall bladder. In both cases the lymph gave strong

tests for bile pigments, much stronger than in any case of simple chronic icterus (see dog 89, p. 129 and dog 91, p. 131).

The experiments with dogs 79 and 86 resemble in practically every detail those with dogs 102 and 112. In dog 79 the flow of lymph was relatively slow and none of the tests of the lymph indicated the presence of bile pigments, although some of them during the second hour were suspicious. In dog 86, in which the flow of lymph was very abundant during the greater part of the observations, we found bile pigments present during the third hour. In both animals, however, chronic icterus developed as usual.

Dog 110.

Urine.			Lymph.	
Time.	Amount.	Bile pigments.	Amount.	Bile pigments.
July 6 Before operation	14 c.c.	Negative.	——	——
1.30 p.m.	Ligation of common bile duct.			
2.20 p.m.	2½ c.c.	Negative.	8½ c.c.	Negative.
2.35 p.m.	3½ c.c.		7½ c.c.	
2.50 p.m.	2 c.c.		7 c.c.	
3.05 p.m.	2½ c.c.		10 c.c.	
3.20 p.m.	1½ c.c.	(Faint) +	9½ c.c.	(Faint) +
3.35 p.m.	3 c.c.		10 c.c.	
3.50 p.m.	3 c.c.	Negative ?	12 c.c.	
4.05 p.m.	3½ c.c.		4 c.c.	
4.20 p.m.	4 c.c.		1 c.c.	
4.35* p.m.	6½ c.c.	Negative	0 c.c.	
4.50 p.m.	7½ c.c.		2 c.c.	
5.05 p.m.	4½ c.c.		——	
5.20 p.m.	4 c.c.	+	6 c.c.	Negative.
5.35 p.m.	2 c.c.		5½ c.c.	
6.00 p.m.	6 c.c.		9½ c.c.	

* Added to fraction of urine 3.05 to 3.35 p. m.

July 6. The dog was a large, white mongrel bull and weighed 30 pounds. At 8 a. m. the animal was given one pound of fat meat which was ground up and mixed with one pint of milk. At 10 a. m. 14 c.c. of urine gave a negative test for bile pigment. Ether anesthesia was begun at 10.40 a. m. and throughout the experiment it was taken poorly. Between 10.40 a. m. and 1 p. m. 1,000 c.c. of 0.8 per cent. salt solution were given subcutaneously. The thoracic duct was isolated and a canula inserted at 11.40. The lymph flow was regular, abundant, and quite milky. In spite of the saline infusion, the flow of urine almost ceased at 1 p. m. At 1.30 p. m. 7 gm. of caffein were given subcutaneously in salt solution. Under the same anesthesia the common bile duct was tied and cut.

The animal came out of the anesthesia at 3.20 p. m. and at 3.30 p. m. vomited a large amount of food. Some of this material was aspirated into the bronchi, and there was cyanosis for several minutes. After 4 p. m. there was an abundant and pretty regular flow of urine. Observations were continued regularly until 6 p. m. In the table that follows are given the results of the urine and lymph tests.

At 6 p. m. the animal was removed to a metabolism cage. Breathing was irregular and secretion from the bronchi was abundant. The next morning, July 7, the animal was found dead in its cage.

Autopsy.—The postmortem changes were pretty well advanced as the weather was very warm and the animal evidently died rather early in the night. Both lungs showed much hemorrhage and a diffuse edema. The bronchi were full of frothy serous fluid. The gall bladder was distended with fluid and definitely enlarged but not very tense. Microscopical sections were of no interest.

Dog 82.

December 27. The animal was a large, fat puppy and weighed 35 pounds. The tests for bile pigment in the urine were negative.

December 28. The dog was given ½ gm. of morphia about half an hour before the operation, and 300 c.c. of water were introduced into the stomach by a stomach tube. During the operation, which was under ether anesthesia, 400 c.c. of saline were given subcutaneously. The common bile duct was doubly *ligated* and cut at 12.15 p. m. The bladder was catheterized every fifteen minutes, beginning at 12.30 p. m., at which time 60 c.c. of clear urine were obtained. Throughout the two hours of observation there was a uniform flow of urine. The urine which was obtained at 12.30 p. m. gave negative tests for bile pigment. The remaining specimens were combined in two portions, one obtained during the first hour and the other obtained during the second hour and were tested with great care for bile pigment but with absolutely negative results. It is possible that the morphia may have influenced indirectly the flow of bile, causing slow accumulation in the bile ducts and gall bladder, a relatively slow rise of pressure, and a tardy escape of bile pigment. The lymph apparatus was not disturbed in this operation.

This experiment, together with others, showed that the time of appearance of bile pigments in the urine varies considerably and is probably dependent in great part upon the activity of bile formation in the liver.

December 29. Tests for bile pigment in the urine were positive.

December 31. The urine presented the usual appearance seen in obstructive jaundice.

January 6 to 9. The dog was losing weight and had a poor appetite. The urine was very highly colored and full of bile.

January 11. At 9.30 a. m. the dog was given 500 c.c. of milk by stomach tube. At 11 a. m. the thoracic duct was exposed, a glass canula was inserted, and a clear canary-yellow fluid was obtained in considerable amounts. Of this clear fluid, 2 c.c. gave a positive test for bile pigment and 12 c.c. gave a positive test for the bile salts. At 3 p. m. the animal was bled to death. The blood was collected in a flask, defibrinated, and centrifugalized. The serum which was obtained had a definite bile tinge and gave positive tests for bile pigments and bile salts.

URINE TESTS.

Time.	Bile pigment.			
Dec. 27	Negative.			
Dec. 28 12.15 p.m.	Common bile duct ligated.			
12.30 p.m.	Negative.			
12.45 p.m. 1.00 p.m. 1.15 p.m. 1.30 p.m.	Negative.			
1.45 p.m. 2.00 p.m. 2.15 p.m. 2.30 p.m.	Negative.			
Dec. 29	+			
Dec. 31	++			
Jan. 11	Lymph		Serum.	
11.00 a.m.	Thoracic duct fistula produced.			
	Bile pigment +	Bile salts +	Bile pigment +	Bile salts +

Autopsy: rupture of gall bladder; peritonitis.

Autopsy.—The peritoneal cavity was full of a yellowish-brown curdy material and of a bile-tinged, unclotted fluid. A rupture had taken place from the gall bladder with an escape of a large amount of bile into the peritoneal cavity. The serous surfaces throughout were covered with a fibrinous exudate and were greatly injected. The mediastinal and mesenteric glands were greatly enlarged, very moist on section, and deeply bile-stained. Heart, lungs, and thorax were normal except for the bile staining of all the tissues. The pancreas was very pale and evidently normal. There were no fat necroses. The cortex of the kidneys was bile-stained. The intestinal tract showed nothing of interest, and the liver had the usual color and general appearance found in obstructive jaundice.

It is reasonable to suppose that a part of the bile and inflammatory exudate in the peritoneal cavity was being removed through the thoracic duct. The strong positive bile pigment test in the lymph is explained in part by this fact and may be compared with that in dogs 86 and 112.

DOG 89.

February 4. The animal was a large brindle bull-dog and weighed about 35 pounds. At 9.15 a. m. the dog drank a quart of milk and at 10.15 a. m. the operation was begun under ether anesthesia. An infusion of 500 c.c. of saline was given subcutaneously, and a canula was inserted into the thoracic duct, but

only a few drops of clear lymph were obtained. The common bile duct was ligated and cut at 12.40 p. m. The secretion of urine after the second operation was very poor and the amounts obtained were insufficient for satisfactory bile pigment tests. At 2 p. m. the dog vomited practically all of the milk which had been ingested. There had been almost no lymph flow from the canula, only a few drops escaping when the animal came out of the anesthesia at about 2 p. m. The urine obtained at 5 p. m. gave a strong test for bile pigment.

February 5. The urine was highly colored and the tests for bile pigment were strongly positive.

February 6. The dog was doing well, but the urine was very highly colored.

February 8. The animal showed a well-developed jaundice, and was killed with ether.

It is of extreme interest to compare the *pericardial fluid*, the *lymph*, the *blood serum*, and the *urine* of this animal. From the pericardial sac two cubic centimeters of a clear, canary-yellow fluid were obtained. The fluid did not clot on standing. The thoracic duct yielded 15 c.c. of a slightly opalescent, canary-yellow lymph in which, on standing, a small clot formed. The serum from the auricle had a clear whiskey color, and the urine from the bladder was tinged a very deep yellow. For the tests for the bile pigments 2 c.c. of each of the four fluids were taken and from these, acidified alcoholic extracts were made, all conditions being as nearly alike as possible. The result of these tests is shown in the following table.

	Thoracic duct fluid.	Pericardial fluid.	Blood serum.	Urine.
Bile pigment test (Salkowski).	+	+	++	++++
Color.	Pale green.	Pale green.	Deep green.	Olive green.

In the alcoholic extracts, the pericardial and thoracic duct fluids had exactly the same tint of pale green, the green of the blood serum was much deeper, and the color of the urine was a deep olive green. The tint of the serum was about twice as dark as that of the thoracic duct fluid, and the color of the urine was very much more intense than that of the serum (compare dog 112).

The autopsy showed nothing of particular interest. The peritoneum was quite clean. The gall bladder and ducts were enormously dilated with tenacious, greenish-black bile. The liver presented the typical appearance found in these experiments. The tissues were all definitely jaundiced. The heart, lungs, spleen, kidneys, and intestines, were all negative.

It seems that this experiment is of some importance as regards the distribution of bile pigments in the different fluids of the body in chronic obstructive jaundice. It may be fair to assume that the concentration of the bile pigments in the fluid in the pericardial cavity, was about the same as that in the lymph and in the fluids of the tissues. The lymph obtained from the thoracic duct contained

almost exactly the same amount of bile pigment as the fluid from the pericardial sac. The concentration in the blood serum was, however, much greater than that in the lymph, indicating that the blood is more active than the lymph in conveying the bile pigments from the liver to the kidneys. The lymph contains bile pigments which have been absorbed from the tissues and the circulating blood and are being returned to the blood by way of the thoracic duct. But in this animal, although the lymph could not gain entrance to the blood stream, the content of bile pigment in the serum was about double that in the lymph. The urine contained relatively greater amounts of the bile pigments as compared with the lymph or serum.

Dog 91.

April 7. The animal was a lean mongrel puppy and weighed 27 pounds. It was quite well and active and drank one quart of milk at 8.30 a. m. Under ether anesthesia at 10.30 a. m. the thoracic duct was exposed and ligated, and from the glass canula inserted in it, a slow flow of lymph was obtained. The lymph was of an opalescent color but not milky. After the second operation the lymph became blood-tinged and remained so throughout the entire observation, more blood being present in the lymph after the animal struggled. In the second operation, performed at once, the common bile duct was doubly ligated and cut at 12 o'clock. During both operations a subcutaneous saline infusion, amounting to 800 c.c. altogether, was given in the axilla and in the groin. The animal came out of the anesthesia at 12.30 p. m. The lymph flow was rather scanty but fairly uniform throughout the entire period. The flow of urine was regular. The lymph and urine were collected every fifteen minutes. The results of the tests of the urine and lymph are given in the following table.

April 8. The animal was well and ate with a good appetite. Only 3 c.c. of urine could be obtained, but in this a test for bile pigment was suspicious. Clean cage urine (20 c.c.) gave a positive test for bile pigment.

April 9. The dog was improving. Only 3 c.c. of urine were obtained by catheterization, but in this the test for the bile pigments was strongly positive. The feces were clay colored.

April 11. The urine was very highly colored and the stools were abundant and clay colored.

April 20. The dog had lost weight steadily, the stools were clay colored, and there was considerable diarrhea.

April 23. The animal weighed 21 pounds. The abdomen was not tender. The dog was killed with ether and bled from the carotids. After allowing the blood to clot in a cylinder, the fluid portion was centrifugalized, and a large amount of perfectly clear serum was obtained. It was amber colored and had a greenish opalescence.

Autopsy.—The subcutaneous fat was scanty and deeply bile-tinged. The

Urine.			Lymph.		
Time.	Amount.	Bile pigments.	Time.	Amount.	Bile pigments.
Before operation	20 c.c.	Negative.	—	—	—
April 7, 12 m. Bile duct ligated.					
12.15 p.m.	3 c.c.		12.15 p.m.	4 c.c.	
12.30 p.m.	3 c.c.		12.30 p.m.	4 c.c.	
12.45 p.m.	4½ c.c.		12.45 p.m.	2½ c.c.	
1.05 p.m.	3½ c.c.		12.50 p.m.	2 c.c.	
1.20 p.m.	4 c.c.	Negative.	1.00 p.m.	2 c.c.	Negative.*
1.30 p.m.	3 c.c.		1.15 p.m.	4 c.c.	
1.45 p.m.	3 c.c.		1.30 p.m.	3 c.c.	
2.00 p.m.	4½ c.c.		1.45 p.m.	3 c.c.	
2.15 p.m.	4½ c.c.	+	2.00 p.m.	3 c.c.	Negative.
2.30 p.m.	3 c.c.		2.15 p.m.	3½ c.c.	
2.45 p.m.	4 c.c.		2.30 p.m.	5 c.c.	
3.05 p.m.	4 c.c.	Negative (?)	2.45 p.m.	4 c.c.	Negative.*
3.15 p.m.	3 c.c.		3.00 p.m.	2½ c.c.	
3.30 p.m.	4½ c.c.	+	3.15 p.m.	1½ c.c.	
3.45 p.m.	4½ c.c.		(Flow ceased)		
April 8	20 c.c.	+	No lymph		
April 9	3 c.c.	++			

* Lymph blood-stained; test not satisfactory.

peritoneal cavity was clear except for old adhesions around the site of the operation upon the common bile duct. The thorax, heart, and lungs were normal, except for deep bile staining; the spleen, pancreas, stomach, and kidneys were also normal. At a distance of 1½ cm. from the pylorus, the duodenum showed a round punched out ulcer which measured 1 cm. in diameter. All the coats of the intestine were involved. It was a typical duodenal ulcer corresponding in every respect to those found in human beings. The rest of the intestine was normal.

The liver showed the usual appearance found in obstructive jaundice. The gall bladder and the cystic and common bile ducts were very large, dilated, and tense, and contained fluid bile. The common duct measured from 1½ to 2 cm. in diameter, but its wall was not greatly thickened. The lymphatics at the hilum of the liver were conspicuous, beaded, and dilated with a clear, yellow fluid exactly like that obtained from the thoracic duct. The pericardial sac contained 2 c.c. of a clear, limpid, pale greenish-yellow fluid. The color was much like that of the blood-serum, but was paler. The thoracic duct was dilated with a clear fluid. On standing, 10 c.c. of this fluid clotted firmly. Equal volumes of blood serum and thoracic duct fluid, when observed in test tubes side by side, appeared of exactly the same color, yet the tests for the bile pigments showed that decidedly more of the pigments were present in the blood serum. The urine contained an enormous amount of bile pigment.

Tests of the urine, serum, lymph, and pericardial fluid were made under identical conditions and resulted as follows:—

	Urine.	Serum.	Lymph.	Pericardial fluid.
Color.	Deep orange.	Clear amber.	Clear amber.	Clear pale greenish yellow.
Salkowski test. .	++++	++	+ (Faint).	+
Color test.	Olive green.	Deep green.	Faint pale green.	Pale green.

This experiment in the acute stage was not as satisfactory as some others, because the rather small amounts of lymph and urine obtained made the tests for the bile pigments less satisfactory. The blood tinge present in the lymph was confusing and made the test rather uncertain. Nevertheless, in this animal, which had a thoracic duct fistula, jaundice developed in quite the usual manner.

The observations upon the body fluids were instructive as they coincided with those upon dogs 89 and 112. These three experiments force one to the conclusion that the blood serum in chronic obstructive jaundice is uniformly richer in bile pigments than the lymph and pericardial fluid. This furnishes more evidence to the effect that the lymphatics take no active part in removing the bile pigments from the liver during the acute or chronic stages of obstructive jaundice.

Dog Y.

February 7. Dog Y was a small, mongrel fox terrier and weighed 13½ pounds. Ten cubic centimeters of urine, tested for bile pigments, were negative.

February 8. Under ether anesthesia the common bile duct was ligated and cut at 10.30 a. m. The animal was in good condition and recovered from the anesthetic almost immediately. After the operation, which lasted only twenty minutes, urine was obtained by catheter and tested for bile pigments with the following result.

Time.	Amount of urine.	Bile pigment.
11.00 a. m.	8 c.c.	+
11.30 a. m.	3½ c.c.	+ (Faint)
12.30 p. m.	3 c.c.	+

This experiment shows that, after the common bile duct is ligated, bile may escape from the liver and appear in the urine almost as rapidly as potassium iodide appeared there after it had been injected into the bile ducts by Mendel and Underhill. The unusually rapid appearance of bile in the urine of this dog may be explained by the shortness of the operation (20 minutes) and by the quickness with which the dog recovered from the anesthetic, for in some of the animals ether was given for two or three hours, the duration varying with the difficulty of the operations.

February 9 to 20. The animal developed typical jaundice, but as it was under observation in connection with work on calcium metabolism there was no further opportunity to examine the body fluids.

From a review of the experiments here recorded, it is clear that our conclusions differ absolutely from those of Heidenhain, Vaughan Hartley, Gerhardt, Eppinger, Herring and Simpson, but that our findings are in accord with those of Wertheimer and Lepage, Mendel and Underhill.

While various foreign substances, potassium iodide, for example, introduced into the bile ducts may be detected in the urine and lymph with far greater accuracy than the bile pigments (this fact possibly explaining some of the minor differences between our results and those of Mendel and Underhill and others), nevertheless we are of the opinion that tests for the bile pigments in the various body fluids are more convincing in studying the path by which, in obstructive jaundice, the bile reaches the kidneys. As our operations were performed under aseptic precautions and as our animals usually recovered, we have been able to make tests for the bile pigments under conditions that closely simulated those of acute and chronic obstructive jaundice. When bile was present in the peritoneal cavity, with or without peritonitis, the fluid in the thoracic duct was found to be rich in bile pigment.

CONCLUSIONS.

These experiments indicate that, in obstructive jaundice, the bile which escapes from the liver is absorbed by the hepatic capillaries and carried by the blood to the kidneys. The presence of a thoracic duct fistula influences in no way the development of icterus after total obstruction of the common bile duct. Bile pigments, sufficient to give a Salkowski test, may or may not appear in the lymph of the thoracic duct in such experiments, their appearance possibly depending upon the rapidity of bile secretion and the amount of lymph flow. Chronic icterus developing in an animal with a thoracic duct fistula gives an interesting distribution of bile pigments in the body fluids. The lymph and pericardial fluid contain the same amount, which is much less than the content of bile pigment in the blood serum and urine. It seems clear that in both

acute and chronic obstructive jaundice the lymphatic apparatus takes no essential or active part in the absorption of bile pigments from the liver. At best, the lymphatic system is a secondary factor in the mechanism of jaundice.

Finally, we wish to express our thanks and appreciation to several who assisted in the difficult and time-consuming operations —B. S. Chaffee, S. H. Hurwitz, O. S. Lowsley, and R. F. Fisher.

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FIBRINOGEN OF THE BLOOD AS INFLUENCED BY THE LIVER NECROSIS OF CHLOROFORM POISONING.[1]

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During the course of some experiments on chloroform poisoning in dogs, it was noted that operations upon many of these animals resulted fatally because of uncontrollable hemorrhage. At autopsy the abdominal cavity was frequently full of fluid blood and, in cases of extreme poisoning, even the blood vessels and heart contained fluid blood and no clots. The present series of experiments was undertaken with the hope of clearing up this feature of chloroform poisoning, but, as the work progressed, it seemed to throw some light on interesting problems connected with blood coagulation and liver function. These experiments seem to indicate clearly that fibrinogen is formed by the liver or that its formation is quite dependent upon the functional activity of that organ.

It will be seen that fibrinogen in the blood can be made to decrease or almost to vanish at will through the production of liver necrosis by chloroform anesthesia. Also, the drop of fibrinogen is found to parallel closely the extent of liver necrosis and, in severe poisoning where the liver shows extensive necrosis, the fibrinogen may be practically absent. In this latter condition, which is not unlike hemophilia, the animal bleeds steadily from large or small cuts. Moreover, the fibrinogen reappears in the blood as the liver begins to repair the injury to its lobules and keeps pace with the repair of the liver cells. The repair is very rapid and may be complete in five or six days. Finally, at the end of the reparatory activity of the liver, we may find an excess of fibrinogen in the blood, an overproduction corresponding to Weigert's law of tissue injury and repair (Chart I).

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It has been shown that the liver is quite capable of repairing the injury done to it by chloroform anesthesia (14). Even when the central necrosis of each lobule involves one-half to three-fifths of all the liver cells, the repair begins on the second or third day and is almost complete in six or seven days. The dead liver cells are removed by autolysis and phagocytosis, and the reticulum is filled in by elongation of the cords of liver cells effected by a rapid mitotic division. The end result is a normal liver. If this mechanism of liver necrosis and repair is kept in mind, it is easy to follow the fluctuation in the fibrinogen content of the blood, as the two are parallel.

The two other factors of blood clotting, thrombin and calcium, have been followed in several of the severe cases of poisoning and are normal. This is in harmony with observations on the character and formation of the blood clots in these cases. Blood drawn from animals poisoned by chloroform anesthesia will clot in the usual time (four to seven minutes), but the clot is more or less flabby, depending upon the amount of fibrinogen present. In severe cases, the clot may be a filmy net-work which will shrink away from the walls of the cylinder and settle to the bottom, forming a very soft, purplish jelly-like mass of not more than five to ten per cent. of the volume of blood. A glass rod can be passed through it with ease, and the threads of fibrin are very delicate and inconspicuous. It is obvious that hemorrhage, which may be a marked feature of these cases, is due to an inefficient blood clot and not to the absence of clotting. It is almost impossible to render an animal's blood quite free from fibrinogen by chloroform poisoning, but it is easy to damage the liver and lower the fibrinogen content to such a degree that the animal will bleed for hours from pricks or cuts in any tissue. It may be said, in general, that these animals do not show a tendency to hemorrhage until the fibrinogen has been reduced 10 per cent. The clot forms in the usual time, but lacks body and stiffness. As the latter characteristic is dependent on the presence of fibrinogen, the clots are too weak and flabby to close the ruptured capillaries and vessels.

A discussion here of the various theories concerning coagulation of the blood is not needed, as excellent reviews of this complicated

subject may be found in the recent writings of Howell (6), Rettger (13), and Loeb (8). We wish to review very briefly some of the work of different observers which is or is not in harmony with our observations and experiments described below.

It has been clearly shown by Doyon and Kareff (2) and by Nolf (11), (12) and his pupils, that extirpation of the liver causes profound changes in the blood, particularly a rapid disappearance of fibrinogen, which may be practically absent at the end of two hours. Nolf claims that the thrombogen also disappears from the blood.

Doyon (3), (4), (5) and his co-workers have reported on several occasions a single experiment with chloroform poisoning. Chloroform was given to the dog by mouth on three consecutive days. The blood obtained one hour before death contained almost no fibrinogen (0.44 per 1,000). Icterus and liver necrosis were present. We have been unable to find any recorded observations, either experimental or clinical, of a very low fibrinogen content of the blood without some accompanying liver injury.

Phosphorus poisoning in animals has added some interesting facts. Corin and Ansiaux (1) have been able to show the absence of fibrinogen in the blood of poisoned animals, and explain the lack of coagulation by this fact. They state that prothrombin and fibrinogen run parallel courses and that both disappear at the same time. Loeb confirmed their findings. They believe that both fibrinogen and prothrombin are formed in the liver. Our observations confirm the findings of Corin and Ansiaux and Loeb in part only, for in our experiments the thrombin remained normal even in the severest cases of chloroform poisoning.

Jacoby (7) states that animals poisoned with phosphorus have no fibrinogen in their blood and that the blood of such animals clots when mixed with normal blood, thus indicating the absence of anticoaguline. We have obtained exactly similar results in chloroform poisoning. Jacoby also states that the injection into a normal animal of the blood of an animal poisoned with phosphorus lowers the coagulation of the latter's blood. In our chloroform experiments we have had a different result, and have shown that, if anything, there is a rise in fibrinogen.

Matthews (9) and, later, Müller (10) found a parallel relationship between the number of leucocytes and the amount of fibrinogen in the blood. Müller injected into animals killed bacterial cultures which produced a rise in fibrinogen in the blood and bone marrow extract, and also a parallel rise in the leucocytes. He concluded that the source of fibrinogen is the leucocyte and that the marrow is a most important factor.

We believe that our experiments exclude the possibility of the marrow's being an important factor and that they show beyond doubt that the white cells are in no way responsible for the fibrinogen of the blood. With the rapid drop in fibrinogen following chloroform poisoning, there is a leucocytosis caused by the liver necrosis and repair. The leucocytosis may be extreme just at the

time when the fibrinogen is lowest. If the liver necrosis is combined with a bronchitis and bronchopneumonia, the white cells may number 320,000 to 330,000 (see table IV, dog c–6), and yet be accompanied by a very low fibrinogen fraction. The bone marrow is never injured by chloroform poisoning and reacts only by a hyperplasia which is the result of the call for blood cells.

METHOD.

Fibrinogen.—To insure a clear plasma, the dogs were starved for twenty-four hours before the blood was taken from the jugular vein by means of a canula. To one part of 1 per cent. sodium oxalate in a cylinder were added nine parts of blood. The oxalated blood was thoroughly mixed and then centrifugalized for about half an hour. As a rule, the perfectly clear plasma, equal to about one-half the total volume, could be removed with a pipette. The oxalated plasma was then carefully acidified with a few drops of 5 per cent. acetic acid, until at last the very faintest acid reaction to litmus was obtained. Of this slightly acidified plasma, 25 c.c. were taken in a pipette and placed in a centrifuge tube. When the amount of fibrinogen was suspected to be low, 50 c.c. of plasma, if available, were taken instead of 25 c.c. The centrifuge tubes were then placed in a water bath whose temperature was kept carefully at 58° to 60° C., and allowed to remain there for ten to twelve minutes. The tubes were then placed in the centrifuge and flocculent precipitate was thrown down completely by prolonged centrifugalization. It is important that the tubes be centrifugalized for a long time at this stage to insure a perfectly clear supernatant fluid. If some of the finer particles are not thrown down, filtration is difficult or impossible, and the procedure becomes very arduous and at times inaccurate. The supernatant fluid is then poured through the weighed filter, and the pure white precipitate is broken up and washed in the centrifuge tubes with an equal volume, 25 to 50 c.c., of cold water.

This mixture was again centrifugalized and the supernatant fluid was poured through the filter. The precipitate was then washed several times with hot water.

After each washing the mixture was centrifugalized and the supernatant fluid was poured through the weighed filter. This procedure was continued until the supernatant fluid gave no precipitate with silver nitrate. Usually three washings will accomplish this and remove all the chlorides. The precipitate was then washed repeatedly with 95 per cent. alcohol and by means of alcohol was carefully washed into the weighed filter. The precipitate on the weighed filter was washed with ether and allowed to dry in the air. The filter was removed carefully to a weighing tube and preserved in a desiccator over sulphuric acid. The filters were dried in an oven at 110° C. for two hours and replaced in the desiccator for at least two hours. The tubes were weighed subsequently at intervals of one to three days until a constant weight, correct within 0.2 mg., was obtained.

It appears that this method is quite accurate and constant under fixed conditions (compare table VI, dog 95). In this instance, two specimens of blood

were taken on the same day from the same animal, examined, and weighed. There was a difference of only 4.4 mg. between the two specimens. In another experiment two fractions from dog 101 were examined on the same day. One specimen of 25 c.c. gave 0.0092 gm. per 100 c.c., and the other specimen of 50 c.c. gave 0.0120 gm. per 100 c.c. It is probable that the latter determination was more nearly correct. When the blood contains such small amounts of fibrinogen, the limits of error are apparently greater than usual.

To test the method further, blood from a normal animal was allowed to clot in a cylinder, the clear serum was rendered very slightly acid to litmus and heated at 60° C. for ten minutes. Prolonged centrifugalization gave no precipitate, indicating that this procedure does not throw down any of the proteids of the blood except fibrinogen. In another experiment the clear supernatant fluid obtained after the first precipitation of fibrinogen by heat and centrifugalization, was again placed in a tube and heated at 58° C. for ten minutes. Prolonged centrifugalization of this specimen gave no precipitate, indicating that the fibrinogen had been totally precipitated by the first heating and completely removed by centrifugalizing.

Calcium.—Measured amounts of blood, 50 c.c. unless otherwise stated, were analyzed for calcium by adding a little nitric acid and evaporating slowly in a porcelain dish until the consistency of a thick syrup was reached. Further evaporation and finally combustion to remove all organic matter, were carried out in a platinum dish. The residue was washed out carefully with a little hydrochloric acid and hot water, boiled in a beaker with sufficient hydrochloric acid to clear up any turbidity, and diluted to 300 to 400 c.c. The whole was neutralized with sodium carbonate until a slight opalescence was obtained. This was cleared again by adding a slight excess of hydrochloric acid. Fifteen grams of ammonium acetate were now added and the whole was boiled for one minute and filtered hot to remove the iron acetate. The precipitate was washed several times with a dilute hot solution of ammonium acetate. The filtrate was evaporated to 200 c.c. and to it were added a small amount of ammonium hydroxide to faint alkalinity and then acetic acid to a faint acid reaction to litmus. On adding 25 c.c. of a 10 per cent. solution of ammonium oxalate, a precipitate of calcium oxalate formed on letting the fluid stand for at least eight hours. The precipitate was collected in the cold on a hard, smooth filter paper, and was washed at least three times with boiling water. The precipitate was then washed into a beaker with hot water, 5 c.c. of concentrated sulphuric acid were added, and the mixture was diluted to 100 c.c. The calcium oxalate was dissolved and the oxalic acid set free. The mixture was then heated to 60° to 65° C. and titrated against a standard solution of potassium permanganate from which the amount of calcium was readily determined.

Thrombin.—The determination of thrombin (Schmidt's method) is only qualitative. In the three estimations of this substance, one of which (dog 85) was made by Dr. W. H. Howell, the method of Schmidt, described by Howell and Rettger, was employed.

Charts.—The fibrinogen curve is indicated by a solid black line, with crosses at the points where the fibrinogen was accurately estimated. Each vertical division represents 0.025 gm. of fibrinogen per 100 c.c. of blood, as indicated on the left hand side of each chart. The calcium curve is indicated by a broken

line and shows little variation except a slight rise, as a rule, when bile pigments were present in the serum. Each vertical division represents 0.002 gm. of calcium per 100 c.c. of blood.

EXPERIMENTS.

The following experiment (dog 104) is summarized, since it is fairly complete and quite typical of all the other experiments. The animal was young, which may explain its great susceptibility to chloroform and the almost fatal result following a single anesthesia of two hours. The poisoning was indicated by a drowsy condition, loss of appetite, repeated vomiting, and especially by the capillary hemorrhages from all cuts. For three days following the chloroform anesthesia, this animal had hemophilia. Blood examination showed no abnormality in its formed elements (Dr. Duke), and the calcium content was normal or above normal. Study of the blood in bulk showed that clotting took place in the normal time, but the clot, instead of being a solid elastic mass, was filmy and not tough and bulky enough to control even capillary hemorrhage. From this evidence, and from that of many other experiments, we are sure that the liver of the dog at this time showed an extensive central necrosis involving a large part of each lobule. Judging from other experiments, we feel sure that liver repair began on the second day and was effective on the fifth day (April 26), for at this time the bleeding stopped and the animal began to improve. Four days later, on the ninth day following the chloroform, operation revealed a liver which had almost completed its regeneration (microscopical section). Blood examination showed a fibrinogen content almost three times as great as before the liver injury, and it is likely that this overproduction resulted from the activity of the regenerating liver cells. Blood examination, after an interval of seventeen days, showed a still higher fibrinogen content, but after a month the fibrinogen had returned to normal.

A second chloroform anesthesia produced in the same dog only a moderate drop in fibrinogen and did not cause the severe symptoms of poisoning observed after the first exhibition of the drug. It is evident that, for some unknown reason, the animal was more resistant to the drug and that the liver was not so gravely injured. It

is possible that the newly formed liver cells are more resistant to the harmful action of chloroform (see also dog c–9), but this increased resistance is not a constant feature.

This experiment in connection with others makes it clear that the fibrinogen of the blood is dependent on liver activity, and that any sudden injury to the liver causes a sudden drop in the fibrinogen. The fibrinogen reappears in the blood in normal or increased amount following the regeneration of the liver cells, and the symptoms of poisoning disappear.

Dog 104.

April 16. Young mongrel hound, female; weight 28½ lbs. Bled under ether anesthesia from jugular vein. The fibrinogen was 0.2192 gm. per 100 c.c., and the calcium was 0.0117 gm. per 100 c.c.

April 17 to 20. Dog recovered well and the neck wound healed rapidly.

April 21, 3 p. m. Chloroform anesthesia for two hours; 1 oz. given. Anesthetic well taken.

April 22. Dog was quite sick and rather drowsy, and vomited repeatedly. Dr. Duke followed the bleeding time of this animal and found it to be greatly delayed. A small ear prick bled for twenty minutes.

April 23. Dog was very quiet and refused to eat. At 11 a. m. it was bled, under ether anesthesia, from jugular vein. The fibrinogen was 0.009 gm. per 100 c.c., and the calcium was 0.0133 gm. per 100 c.c. The blood clot was very flabby and, on standing, settled at the bottom of the graduate. Its volume was about 10 per cent. of the blood in the graduate, and offered scarcely any resistance to a glass rod. The plasma obtained after centrifugalization was a clear amber. The animal bled, drop by drop, from the operative wound in the neck during the course of the entire day. A bandage was tied tightly about the neck, but did not suffice to check the flow. The cloth became wet with blood, but there was no clotting.

April 24. Animal was very sick and weak. Bleeding continued and the blood did not clot. Animal given one-fourth grain of morphia subcutaneously.

April 25. Dog was very weak and anemic. The pulse was irregular and the bandage was completely blood-soaked. On removing the bandage the wound continued to ooze blood, drop by drop. There were no clots in the wound or on the bandage.

April 26. In the morning some fluid blood was found under the cage, but the neck wound had stopped bleeding and was distended with a very soft, purple, loosely adherent blood clot. Hemoglobin was 60 per cent. which is about half the normal quantity for a dog.

April 27 and 28. Dog was improving slowly and began to eat.

April 29 and 30. Animal was gaining in weight and eating vigorously. Weight 23 lbs. The blood showed normal coagulation and bleeding time, but signs of active regeneration. There was a leucocytosis, and nucleated red blood cells were numerous in the circulation.

Operation.—At 10 a. m. one-third grain of morphia was given subcutaneously. Ether anesthesia. Incision was made through the right rectus, and the liver was exposed with a little difficulty. The organ was very pale, but the lobulation was quite regular and apparently normal. The centers of the lobules were rather conspicuous and appeared as rusty specks, a condition usually found in the last stages of liver regeneration following the central necrosis of chloroform poisoning. The blood clots perfectly and effectually in the liver wound, and there are no operative difficulties. At the end of the operation the animal was bled and the fibrinogen was found to be 0.6130 gm. per 100 c.c.

Liver.—Microscopical sections. The central fifth of each liver lobule showed a few fat droplets of varying size situated in the liver cells. No necrosis was seen, as all the necrotic debris had been dissolved and removed. The repair was almost complete, but there was an empty reticulum in the very center of each lobule. In this reticulum were a few polymorphonuclear cells and quite a number of large mononuclear phagocytes which contained finely granular, yellow pigment. Mitotic figures were not found, indicating that the active multiplication of the liver cells was completed. Many of the bile canaliculi were dilated with small, yellowish-green hyaline casts.

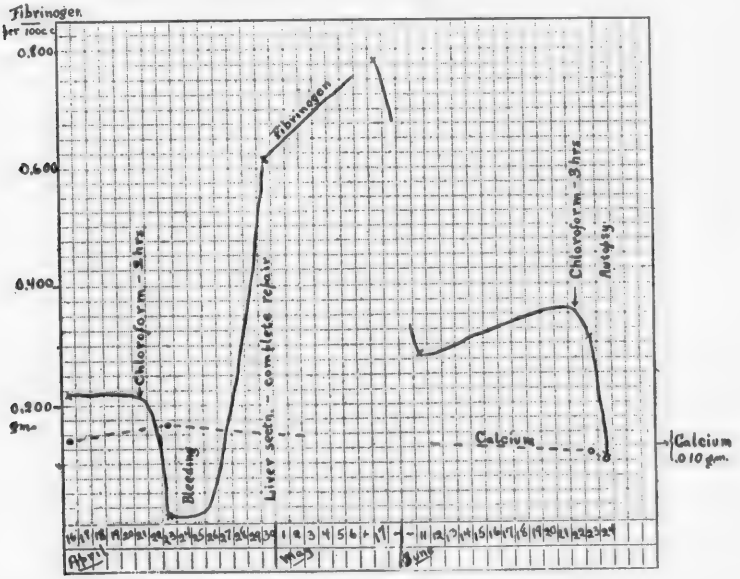

CHART I. Dog 104.

May 17. Animal was pretty active. It was bled from jugular vein under ether anesthesia. The fibrinogen was 0.7800 gm. per 100 c.c.

May 21. Animal did not eat well and had signs of distemper.

June 11. Animal bled, under ether anesthesia, from jugular vein. The fibrinogen was 0.2848 gm. per 100 c.c.

TABLE I. Dog 104.

Date.	Chloroform.	Fibrinogen in 100 c.c.	Calcium in 100 c.c.	Platelets.	White blood corpuscles.	Jaundice.	Bleeding.
April 16	—	0.2192 gm.	0.0117 gm.	459,000	22,400	—	—
April 21	2 hrs.	—	—	236,000	5,270	—	—
April 22	—	—	—	321,000	11,000	?	+
April 23	—	0.0090 gm.	0.0133 gm.	158,000	8,900	+	+++
April 26	—	—	—	186,000	55,000	+	—
April 30	—	0.6130 gm.	—	270,000	50,000	—	—
May 17	—	0.7800 gm.	—	—	—	—	—
June 11	—	0.2848 gm.	—	—	—	—	—
June 22	3 hrs.	—	—	—	—	—	—
June 23	—	0.3072 gm.	0.0090 gm.	—	—	?	—
June 24	—	0.100± gm.	0.0087 gm.	—	—	+	+
June 24		Autopsy: liver necrosis involved over two-fifths of each lobule.					

June 22. Dog 104 was quite well; weight 24 lbs. At 9.25 a. m. chloroform anesthesia for three hours; 1½ oz. given. The anesthesia was very light and well taken; recovery rapid.

June 23, 11 a. m. Animal seemed quite well and was apparently not poisoned as by the previous anesthesia. It was bled, under ether anesthesia, from jugular vein. Fibrinogen was 0.3072 gm. per 100 c.c. The plasma, after centrifugalization, had a clear pale lemon color. The calcium was 0.009 gm. per 100 c.c.

June 24. Animal would not eat, but seemed pretty well. There was only a little oozing from the neck wound and no vomiting. Bled to death at 4 p. m. under ether anesthesia from carotid artery. Calcium and fibrinogen determined as usual. Serum had a clear amber color and contained bile pigments in considerable amounts. The clots which formed in the graduate were decidedly soft and flabby.

Autopsy.—Performed at once. The serous cavities were all normal except for a few old adhesions about the site of operation in the liver. Heart, lungs, spleen, pancreas, stomach, duodenum, and kidneys were normal, gross and microscopically.

Liver.—Decidedly pale. The lobulation was distinct, and in the centers of the lobules were seen collapsed pink areas, while the portal tissue was quite opaque and yellow, the usual picture in chloroform poisoning. The organ was large, rather tense, and friable. Microscopical sections showed a moderate grade of hyaline cell necrosis involving the central two-fifths of each lobule. The necrotic areas were invaded by a good many wandering cells and were surrounded by a thin zone of fatty degeneration, in which all of the liver cells contained large and small fat droplets. About half of the liver cells were uninjured and appeared practically normal. Numerous mitotic figures were present in the boundary zone. The very centers of the lobules showed a few large, pigmented mononuclear phagocytes—all that remained to indicate the first chloroform poisoning, from which recovery had been complete. The bile ducts were all normal.

Dog 81.

May 5. Large black and white female; weight 44½ lbs. Bled, under ether anesthesia, from jugular vein. Fibrinogen was 0.2972 gm. per 100 c.c., and calcium was 0.009 gm. per 100 c.c.

May 10. Dog was quite well.

May 11. Chloroform anesthesia for two hours; 1¾ oz. given.

May 12. Dog vomited repeatedly and drank much water. It was thought best not to give more chloroform, as the animal appeared to be too sick.

May 13. Chloroform anesthesia for two hours; 1¾ oz. given. As the animal was coming out of the anesthesia, she vomited about 75 c.c. of blood and blood clots, but did not seem dangerously ill. At 6 p. m. the dog seemed very sick.

May 14. At 10 a. m., dog was bled from jugular vein under ether anesthesia. The serum had a clear amber color, the fibrinogen was 0.002 gm. per 100 c.c., and the calcium was 0.0097 gm. per 100 c.c. At 3 p. m. the dog was bleeding drop by drop from the neck incision, and about 50 c.c. had collected in the container under the cage. The dog seemed very sick.

May 15. About 100 c.c. of blood had escaped during the night, due to a steady oozing from the small neck wound. Bleeding stopped for a time during the forenoon, but the animal was still very sick.

May 16. The wound bled again during the night, for about 50 c.c. of unclotted blood was found in the container.

May 17. Dog began to eat and was evidently improving.

May 21. Animal was practically normal and weighed 41¼ lbs.

June 16. Dog was bled from jugular vein; the fibrinogen was 0.2984 gm. per 100 c.c. and the calcium was 0.010 gm. per 100 c.c.

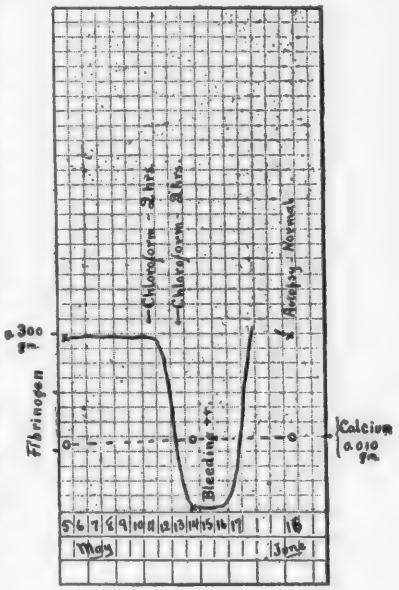

CHART 2. Dog 81.

June 30. Weight 42 lbs. Animal killed with ether; autopsy performed at

TABLE II. Dog 81.

Time.	Chloroform.	Fibrinogen in 100 c.c.	Calcium in 100 c.c.	Platelets.	Jaundice.	Bleeding.
May 5	—	0.2972 gm.	0.009 gm.	—	—	—
May 11	2 hrs.	—	—	—	—	—
May 13	2 hrs.	—	—	179,000	?	(stomach)
May 14	—	0.0020 gm.	0.0097 gm.	188,000	+	++
May 15	—	—	—	—	+	++
May 16	—	—	—	—	?	+
May 17	—	—	—	—	—	—
June 16	—	0.2984 gm.	0.010 gm.	—	—	—
June 30	Autopsy: liver normal.					

once. There was an extreme grade of chronic pancreatitis, but the other organs were normal.

Liver.—Microscopical sections showed that the liver repair was complete. In the centers of some of the lobules were seen a few pigmented phagocytes. The architecture was perfectly normal.

Dog 85.

January 13. Black mongrel female pup; weight 20 lbs. Chloroform anesthesia for two hours; ⅜ oz. given. The anesthetic was well taken.

January 14. Dog was quite active and appeared well. Urine contained bile pigment. Chloroform anesthesia for one and one-half hours; ½ oz. given.

January 15. Dog seemed a little dull. No vomiting. Urine contained a large amount of bile pigment. 11 a. m., operation under ether anesthesia. Incision was made through the right rectus. The liver was exposed and a small wedge-shaped piece was removed. The lobulation was conspicuous and there was evidently a good deal of necrosis. At the site of operation there was almost no clotting of the blood.

Thrombin.—The dog was bled to death at once from the carotid for the determination of thrombin. In a graduate 100 c.c. of blood clotted in the normal time. After one hour the clot retracted and settled at the bottom of the graduate. Its volume was 5 c.c. The clot was almost semifluid and offered no resistance to the passage of a glass rod through it. After centrifugalization the serum had a perfectly clear lemon color and contained a large amount of bile pigment. Fifty cubic centimeters of the clear serum were precipitated with twenty volumes of 95 per cent. alcohol and were examined after a period of six weeks by Dr. W. H. Howell, who demonstrated that thrombin, in normal amount, was present.

Autopsy.—The autopsy was performed at once. The serous cavities were normal; the heart and lungs showed nothing of interest; the thymus was full of small ecchymoses; and the pancreas, stomach, duodenum, and kidneys were all normal.

Liver.—The liver showed the characteristics seen in severe chloroform poisoning. It was swollen, tense, and very friable. The lobulation was conspicuous. The centers of the lobules appeared as bright red dots. The liver tissue elsewhere was opaque and yellow. Microscopical sections showed that nearly all the cells had undergone hyaline necrosis. One or two rows of relatively normal liver cells remained about the portal structures.

If we judge from other experiments, it is very unlikely that this animal would have recovered from such an extreme degree of liver necrosis. Unfortunately no accurate determination of fibrinogen was made in this case, but from other experiments we may be sure that it was reduced to a minimum.

At this point, we may refer again to the papers of Nolf, Loeb, and Corin and Ansiaux, who are convinced that the thrombin or

prothrombin has its origin in the liver or, at least, is dependent upon liver activity for its presence in the blood. The last experiment and the two that follow speak strongly against this view, for in these we have extreme chloroform poisoning with liver necrosis involving almost all the cells in each lobule and with the merest trace of fibrinogen in the blood, yet the thrombin was present.

Dog 88.

January 28. Large pregnant female; weight 47 lbs. Chloroform anesthesia for two hours; 1 oz. given. During the anesthesia, incision through the median line, exposure of the pregnant uterus, and removal of a part of the left uterine horn, in which a good-sized fetus was contained.

January 29. Animal appeared quite well.

January 30. Condition remained the same. Urine contained a large amount of bile pigment.

January 31. Animal was quite well. Chloroform anesthesia for two hours; 1¼ oz. given. The anesthetic was not well taken and muscular tremors were marked. During the anesthesia, an operative incision was made through the right rectus with exposure of the right uterine horn. A large part of this was resected, two fetuses being removed. Due to placental separation, there had been some intra-uterine hemorrhage. At the time of operation there was much bleeding because of inefficient clotting.

February 1. Animal looked weak. At 2 p. m. animal vomited mucus and water and appeared quite drowsy. Under anesthesia the dog was bled from the carotids and a large amount of blood was collected for the study of thrombin. After the blood clotted, the serum was centrifugalized and 50 c.c. of the clear amber-colored fluid was removed and precipitated with 950 c.c. of 95 per cent. alcohol. After standing for five months the precipitate was collected, dried, tested as described above, and *thrombin* was demonstrated to be present. The blood obtained from this dog clotted in the usual time, but the clots were small and very flabby, breaking up when simply poured out of the cylinder.

Autopsy.—Heart, lungs, spleen, stomach, and intestines were normal. The uterus contained six fetuses. Of these one was alive when removed from the uterus, but three were partly macerated, due to placental hemorrhage and separation. Placental necrosis, hemorrhage, and separation are the rule in pregnant dogs after chloroform anesthesia.

Liver.—In gross, the liver presented the usual appearance. On microscopical section, all the lobules showed central necrosis involving about one-fourth to two-fifths of the liver cells. The remaining liver cells showed an extreme grade of fatty change and were packed with large and small fat droplets. Mitotic figures were not very numerous.

Dog 101.

March 19. Old mongrel hound, male; weight 41½ lbs. Animal was bled, under ether anesthesia, from jugular vein. The fibrinogen was 0.3316 gm. per 100 c.c. Serum was clear as water. Chloroform anesthesia for one and one-

half hours; 1¼ oz. was given. In the afternoon the dog seemed badly poisoned.

March 20. Animal was very quiet and would not eat. Chloroform anesthesia for one hour; 1 oz. was given. The anesthetic was well taken.

March 21. Chloroform for one and three-fourth hours; 1¾ oz. was given.

March 22. Dog seemed pretty well. Under ether anesthesia the dog was bled from carotid. Part of the blood was obtained in oxalate for the determination of fibrinogen. Two different fractions were weighed in order to test the method. One fraction of 50 c.c. gave a fibrinogen content of 0.0120 gm. per 100 c.c. The other fraction of 25 c.c. gave a fibrinogen content of 0.0092 gm. per 100 c.c. The serum was colored a perfectly clear amber. Fifty cubic centimeters of blood were analyzed for calcium and gave a normal amount (0.0108 gm. per 100 c.c.). Thirty cubic centimeters of clear amber-colored serum were analyzed for calcium and gave 0.0083 gm. per 100 c.c. A large amount of blood was received into cylinders and clotted in the usual time. The blood clots were very tenuous and soft. They settled on standing into soft purple masses, occupying 10 to 20 c.c. per 100 c.c. of blood. These clots offered practically no resistance to a glass rod. In order to determine the thrombin, 50 c.c. of clear serum were added to 950 c.c. of 95 per cent. alcohol. The thrombin was subsequently shown to be present.

TABLE III. Dog 101.

Date.	Chloroform.	Fibrinogen in 100 c.c.	Calcium in 100 c.c.	Thrombin.	Jaundice.
March 19	1½ hrs.	0.3316 gm.	—	—	—
March 20	1 hr.	—	—	—	+
March 21	1¾ hrs.	—	—	—	+
March 22	—	0.0092 gm.	(blood) 0.0108 gm.	+	++
March 22	—	0.0120 gm.	(serum) 0.0083 gm.		
March 22	Autopsy: liver necrosis invaded one-half of each lobule.				

Autopsy.—The autopsy was performed at once. Thorax, lungs, and spleen were normal. The heart contained one large filaria in the conus of the right ventricle. In the pancreas there were a few small inconspicuous fat necroses just below its peritoneal covering and uniformly distributed throughout all its parts. The medullary rays of the kidneys contained many tubules which were full of fat droplets. The condition was more marked than in the normal dog's kidney.

Liver.—The organ was considerably enlarged. The centers of the lobules were bright red and rather sunken. The periphery of each lobule was translucent, but the middle zone was decidedly opaque and yellow. There were no hemorrhages. On microscopical section each lobule showed evidence of a central necrosis involving about one-half of the cells. Much of the necrotic material had been dissolved and removed, leaving an empty reticulum containing a good many phagocytes and dilated capillaries. Many of the dead liver cells remained as spherical or irregular oval granular hyaline masses in which

no nuclei were seen. Polymorphonuclear cells were not very numerous. The liver cells about the portal spaces were much swollen and contained numbers of fat vacuoles. Mitotic figures were very numerous, and repair by the remaining intact liver cells was evidently going on with great rapidity. The portal structures and bile ducts were normal.

The bone marrow of the femur was red and cellular in places, but was in great part made up of fat. Microscopical section showed a definite grade of patchy hyperplasia. This had involved particularly the polymorphonuclear leucocytes and their precursors. There was no evidence of cell injury. The giant cells of the bone marrow were fairly numerous and quite normal in appearance.

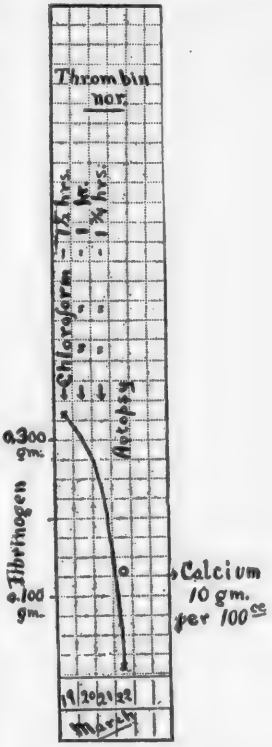

CHART 3. Dog 101.

DOG C–6.

April 12. Mongrel male; weight 24¾ lbs. Chloroform anesthesia for two hours; 1 oz. given. The anesthetic was well taken.

April 13. Chloroform anesthesia for two hours; 1 oz. given.

April 14. Chloroform anesthesia for one hour; ½ oz. given. The anesthetic was poorly taken. The animal bled easily from an ear prick, and the bleeding continued for twenty to thirty minutes.

April 15. From small ear pricks which were still bleeding, the dog had lost during the night about 20 to 30 c.c. of blood. No clots were formed at the edges of these small incisions. The animal appeared weak and restless, but did not vomit. At 10 a. m. under ether anesthesia the dog was bled from the carotid. The serum was colored a clear amber. The fibrinogen was 0.0184 gm. per 100 c.c. and the calcium was 0.0141 gm. per 100 c.c. Blood which was collected in cylinders clotted in the usual time, but the blood clots were very flabby and settled rapidly to the bottom. The clots constituted about 10 per cent. of the volume in the cylinder.

Table IV (dog C–6) shows a remarkable leucocytosis, and these counts may be considered accurate because of repeated examinations to confirm the unusual rise from normal to over 300,000 white blood cells.

Autopsy.—The autopsy was made immediately after death. The serous cavities were normal, as were also the heart, spleen, kidneys, stomach, and intestines.

Lungs.—Both the lower lobes of the lungs showed greenish patches of consolidation. In the pleura, over these areas, were numerous ecchymoses. The lung tissue was decidedly moist. In the microscopical section were patches

TABLE IV. Dog C-6.

Time.	Chloroform.	Fibrinogen in 100 c.c.	Calcium in 100 c.c.	Platelets.	White blood cells.	Bile pigment.	Bleeding.
April 12	2 hrs.	—	—	220,000	30,000	—	—
April 13	2 hrs.	—	—	434,000	62,000	+	—
April 14	1 hr.	—	—	Abundant in smears	320,000	+	++
April 15	—	0.0184 gm.	0.0141 gm.	Abundant in smears	330,000	++	+
April 15	Autopsy: liver necrosis invaded about two-fifths of each lobule. Normal cells were few.						

of bronchopneumonia in which polymorphonuclear leucocytes and red blood cells were the predominant features. Very little fibrin was present. It was compact, glassy, and hyaline, as though of considerable age. No strands of fresh fibrin were present.

The pancreas was quite normal. The bone marrow (femur) had a mottled yellowish-pink color. Microscopical section showed an active hyperplasia affecting particularly the polymorphonuclear leucocytes. The bone marrow giant cells were numerous, but of rather small size. There was no cell necrosis and no nuclear fragmentation.

Liver.—The liver was large, tense, and friable. The lobules were very distinct with bright red centers and with thin, swollen, opaque margins. In microscopical sections, each lobule showed evidence of a central hyaline necrosis involving about two-fifths of the cells. There was a well-marked middle zone in which the liver cells contained large and small fat droplets. This zone was estimated to be about one-fifth of the volume of each lobule. The cells immediately surrounding the portal structures were practically normal, and mitotic figures were very numerous. Mononuclear phagocytes and polymorphonuclear cells were very numerous in the areas of necrosis.

Dog C-9.

April 26. Large mongrel male pup; weight 23 lbs. Chloroform anesthesia for two hours; ¾ oz. given; anesthetic well taken.

April 28. The dog was quite sick and vomited frequently. Urine contained a large amount of bile pigment.

April 29. Dog was quite sick. Bleeding time was prolonged to four to eight minutes.

April 30. There was a little improvement. Under ether anesthesia the dog was bled from jugular vein. The fibrinogen was 0.1504 gm. per 100 c.c.

May 1. Dog was not well and would not eat.

May 10. Improvement had been slow; weight 19 lbs.

May 17. Under ether anesthesia the animal was bled from jugular. The fibrinogen was 0.6820 gm. per 100 c.c.

June 9. Dog was well. Weight 22 lbs.

June 13. Chloroform anesthesia for two hours; 1 oz. given; anesthetic well taken.

TABLE V. Dog C–9.

Time.	Chloroform.	Fibrinogen in 100 c.c.	Calcium in 100 c.c.	Platelets.	Jaundice.	Bleeding.
April 26	2 hrs.	—	—	—	—	—
April 27	—	—	—	—	+	—
April 28	—	—	—	—	++	—
April 29	—	—	—	228,000	?	+
April 30	—	0.1504 gm.	—	306,000	+	—
May 17	—	0.6820 gm.	—	—	—	—
June 13	2 hrs.	—	—	—	—	—
June 14	2 hrs.	—	—	—	?	—
June 15	2 hrs.	—	—	—	++	—
June 16	—	0.100± gm.	0.0102 gm.	—	++	—
June 16	Autospy: liver necrosis invaded one-fourth of each lobule. Very active repair.					

June 14. Animal appeared well; no vomiting. At 11 a. m. chloroform anesthesia for two hours; 1½ oz. given.

June 15. Animal refused food at 3 p. m.; chloroform for two hours; 1½ oz. given.

June 16. Dog appeared pretty well. Urine contained a large amount of bile pigment. At 2 p. m. the animal was killed accidentally at the beginning of anesthesia.

Autopsy.—The autopsy was performed immediately after death. Unclotted blood was obtained from the right auricle and mixed with oxalate solution, as usual, for the determination of fibrinogen. This amounted to about 0.100 gm. per 100 c.c. The serum was canary yellow in color and slightly milky, due to an alimentary lipemia. Blood drawn into cylinders clotted in the usual time and pretty firmly. The clots were broken up with considerable ease, but retained their shape well and did not shrink. Extreme reduction in fibrinogen was not suggested. One hundred cubic centimeters of blood, analyzed for calcium, yielded 0.0102 gm.

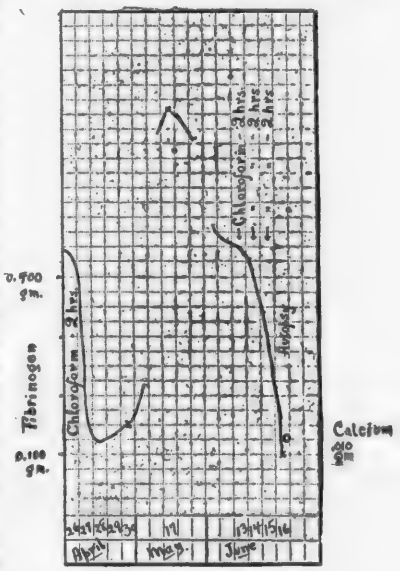

CHART 4. Dog C–9.

The heart, spleen, kidneys, stomach, duodenum, intestines, and pancreas were normal. In the lungs were large areas of consolidation having a greenish red color. They measured 1 or 2 cm. in diameter and were present particularly in the lower lobes. The bronchi were full of creamy material. The pleural surface was smooth and moist. On microscopical examination the

bronchi were found to be full of an exudate made up in great part of well preserved polymorphonuclear leucocytes. The alveoli were full of an exudate of polymorphonuclear cells, mononuclear cells, a few red blood cells, and a granular coagulum.

Liver.—The liver was large and friable. As usual, the lobulation was conspicuous. Microscopically the liver necrosis was not very striking in this case. This is rather surprising considering the duration of chloroform anesthesia. More than half of the liver cells in each lobule were uninjured or, at most, showed only a few small fat droplets. Mitotic figures were numerous, and evidently the liver cells repaired the injury very effectually and with great rapidity. The centers of the lobules showed almost complete removal of the necrotic liver cells. A few small hyaline masses remained, some of which were undergoing calcification. Large pigmented phagocytes were numerous.

The resistance of this animal (dog c–9) to chloroform anesthesia is difficult to explain. It is evident, however, that its liver was very resistant to the drug and that the injury was repaired with greater rapidity than normally. The findings in the liver sections corresponded exactly with the fibrinogen content of the blood, which, although considerably reduced, was not dangerously low. Undoubtedly the bronchitis and bronchopneumonia were the most important factors in the animal's death.

In the preceding experiments several interesting points are brought out. The chart and table of dog 101 show that chloroform anesthesia given at short intervals, can reduce the fibrinogen fraction of the blood to a minimum, causing at the same time extensive liver necrosis. The experiment with dog c–6 shows much the same as regards the fibrinogen, and supports the other observations. In two dogs (Nos. 85 and 88) the thrombin and calcium were found to be normal. In dogs 104 and c–9 the formed elements of the blood, especially the platelets, were not involved except in a secondary manner, for the liver necrosis calls for many leucocytes. This caused a rise in the white cells in the blood for two to four days following the liver injury, and effected some hyperplasia of the bone marrow. The bone marrow giant cells, as well as all the other marrow cells, respond to this hyperplasia, and platelets are more numerous than normally in the circulating blood. The bone marrow shows no signs of injury by the drug.

Table IV (dog c–6) is of particular interest in connection with

the view of Müller that fibrinogen is produced by the leucocytes. In this experiment the chloroform anesthesia caused extensive liver necrosis and reduced the fibrinogen content of the blood to about 5 per cent. of the normal. Instead of a leucopenia we had a tremendous leucocytosis due probably to the combination of liver necrosis and bronchopneumonia, which stimulated the bone marrow to hyperactivity. It is difficult to explain the great decrease in fibrinogen which took place in this case simultaneously with the tremendous increase in the leucocytes, if the leucocytes are in any way concerned with the production of fibrinogen.

Chart 4 and Table V (dog c–9) show a fibrinogen curve similar to that in Chart I. About six weeks after the first anesthesia, this animal manifested a greater resistance to chloroform than in the beginning. The liver was able to repair the injury very actively; the blood contained a fair amount of fibrinogen (0.100 gms. ± per 100 c.c.); the dog gave few signs of poisoning; and it did not bleed from small cuts. This observation suggests again the possibility that the primary injury to the liver conferred some immunity. Nevertheless, animals vary so greatly that, to establish this point, much evidence must be accumulated.

Dog c–3.

April 7. Old mongrel, male; weight 64 lbs. Chloroform anesthesia for two hours; 1½ oz. given. During the anesthesia 200 c.c. of blood were obtained from the jugular vein and 400 c.c. of 0.8 per cent. salt solution, containing 16 gms. of calcium lactate, were infused subcutaneously. The fibrinogen determination was 0.4492 gm. per 100 c.c.

April 8. Dog was very sick, and at 3.30 p. m. appeared to be fatally poisoned. The animal was bled under ether anesthesia from the carotid. The fibrinogen was 0.2340 gm. per 100 c.c. and the calcium was 0.008 gm. per 100 c.c. The blood drawn in cylinders clotted rather slowly, but the clot was pretty firm. A large amount of blood was defibrinated in a flask, and 180 c.c. of this defibrinated blood was injected intravenously into dog C–4.

Autopsy.—This was performed at once. All the tissues showed a delicate icteroid tint. The serous cavities, as well as the heart, lungs, spleen, and kidneys, were normal. In all parts of the parenchyma of the pancreas were great numbers of fat necroses, some of the larger ones measuring 2 mm. in diameter. There were no hemorrhages.

Liver.—A large amount of blood was present, giving the liver a deep purple color. The lobules were sharply outlined. In microscopical sections all the liver cells contained fine fat droplets, which were of larger size and more con-

spicuous in the central part of each lobule. There was extensive necrosis involving about one-half of each lobule. The necrosis was quite recent (thirty hours), and the liver cells retained their contour very well. The protoplasm, however, in these cells was rather glassy and uniform, and the nuclei showed pyknosis and fragmentation. There were very few wandering cells. The capillaries were congested.

Dog c–4.

April 8. Fox terrier, male; weight 16 lbs. Under ether anesthesia 100 c.c. of blood were obtained from the jugular vein and infused intravenously with 180 c.c. of defibrinated blood from dog C–3. The animal was under anesthesia for only twenty minutes and recovery was rapid. Fibrinogen was determined (0.6040 gm. per 100 c.c.). There was a little vomiting during the night.

April 9. Dog was quite lively, but did not eat.

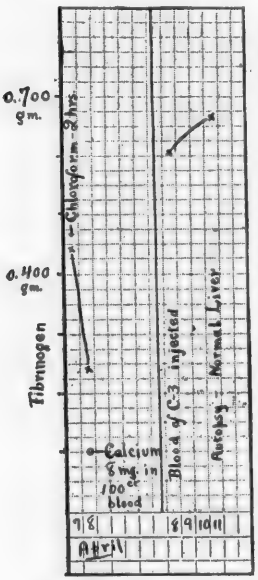

Chart 5. Dog C–4.

April 11. Dog was improving; was killed with ether. The fibrinogen determination gave 0.6712 gm. per 100 c.c. The thorax, heart, lungs, and spleen were normal. The peritoneal cavity showed a little fresh fibrinous exudate over the dome of the liver and particularly about the gall bladder, which had a deeply injected, purplish, rough, serous covering. There were a good many ecchymoses in this region. The gall bladder contained normal bile, and the mucosa showed no change. The rest of the peritoneal cavity was practically normal. The pancreas and kidneys were normal, and the stomach and intestines were full of food and showed a normal mucosa.

Liver.—The liver lobulation was definite and rather conspicuous. The lobules seemed somewhat swollen and the centers were congested. In the microscopical sections, all of the liver cells contained a few tiny fat droplets which were brought out only by fat stains. There was no evidence of cell necrosis.

We are unable to explain the early peritonitis, which, however, was not of a severe grade and did not prevent the animal from taking food. It is possible that this followed the introduction of bacteria at the time of the infusion. This experiment, together with others to be reported later, indicates beyond any doubt that blood removed from an animal poisoned with chloroform is not poisonous to another animal when given intravenously. The slight rise in fibrinogen may be explained by the

peritonitis which had begun, for it is known that the fibrinogen content of the blood is high in pneumonia, peritonitis, etc.

Dog 96.

March 5. Small young female; weight 17 lbs. The dog was bled, under ether anesthesia, from the jugular vein. The fibrinogen was 0.3120 gm. per 100 c.c.

March 10. The dog is quite well and is bled again, under ether anesthesia. The fibrinogen was 0.2872 gm. per 100 c.c.

May 4. Chloroform anesthesia for one hour and fifty minutes; ¾ oz. given.

May 5. Animal bled from jugular vein, under ether anesthesia. The fibrinogen was 0.0150 gm. per 100 c.c.

May 6. Animal found dead in cage, the result of bleeding from the neck, which, however, contained some very soft clots.

Autopsy.—The serous cavities, lungs, spleen, and kidneys were normal. The heart was full of purple, soft, almost semi-fluid clots.

Liver.—The liver showed the usual characteristics seen in chloroform poisoning. In the microscopical sections was found an extreme grade of central necrosis involving more than half of every lobule. Only three or four rows of normal liver cells surrounded the portal spaces. There was a thin mid-zone of fatty degeneration. The mitotic figures were very few. Evidently this dog was severely and probably fatally poisoned by the single administration of the anesthetic. Examination of the blood showed a remarkably rapid and almost complete disappearance of the fibrinogen at the end of twenty-four hours. This in itself indicated a very high grade of poisoning, as the usual diminution at the end of twenty-four hours is not more than 50 per cent.

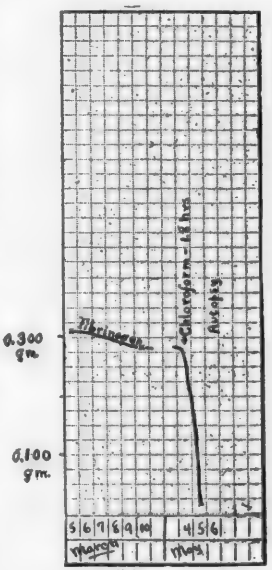

Chart 6. Dog 96.

Dog 92.

February 22. Fox terrier, male pup; weight 17 lbs. Bled, under ether anesthesia, from jugular vein. The fibrinogen was 0.7896 gm. per 100 c.c. It is possible that this determination is too high as it was one of the earliest in the series, and the determination was made without the use of the centrifuge to throw down the precipitate.

March 15. Weight 16½ lbs. The dog was bled, under ether anesthesia, from the jugular vein into oxalate solution. The fibrinogen was 0.5396 gm. per 100 c.c.

May 13. Chloroform anesthesia for two hours; ¾ oz. given.

May 14. The dog was bled, under ether anesthesia, from jugular vein. The fibrinogen was 0.3776 gm. per 100 c.c.

May 15. The animal seemed quite well.

June 22. Chloroform anesthesia for two hours; 1 oz. given. During this time the blood of the animal was defibrinated. Canulas were inserted into the carotid artery and jugular vein. About 200 to 300 c.c. of blood were allowed to flow into a defibrinating flask. The blood was defibrinated by shaking with beads; it was then strained through gauze, and allowed to flow back slowly into the jugular vein. The defibrinating bottle was then rinsed out with a little 0.8 per cent. salt solution, and the rinsing solution, also, was introduced through the canula. This procedure was repeated six times during the two hours of anesthesia. The blood removed at the last bleeding remained fluid, and practically no fibrin was formed after shaking for five minutes. No clot formed in the carotid canula during a period of ten minutes after the vessel was clamped, and it is pretty clear that the circulating blood contained very little fibrinogen.

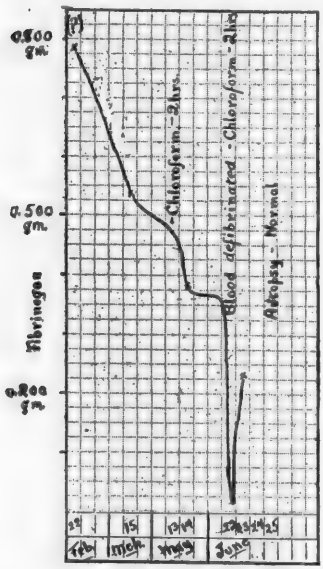

CHART 7. Dog 92.

June 23. The dog was very quiet, but did not look particularly ill. At 11 a. m. the bandages were removed and some large soft clots were found in the neck wound from which a little blood was still oozing. Under ether, the animal was bled from the same carotid into oxalate solution. The fibrinogen was 0.2368 gm. per 100 c.c. The serum had a clear rose pink color due to hemolysis. The volume of the red cells was about one-half the normal for, after centrifugalization, the red blood cells made up only about one-fourth of the total volume. Urine obtained during this time showed a definite hemoglobinuria resulting from the hemolysis and hemoglobinemia.

June 25. The dog ate well and appeared to be in good condition. It was killed with ether.

Autopsy.—The autopsy was performed at once. The serous cavities, heart, lungs, spleen, pancreas, duodenum, and stomach, were perfectly normal. The kidneys showed a pale greenish coloration of the cortex, but were otherwise normal.

Liver.—The liver was practically normal. Microscopical sections showed no fatty cell changes or necrosis.

We are not prepared to explain the lack of necrosis observed in the liver following chloroform anesthesia. This is an isolated observation and evidently has to do with the defibrination procedure. It is possible that the repeated withdrawals of the blood

permitted the escape of much of the chloroform which it contained, for, presumably, the anesthetic injures the liver by circulating through it. Enough chloroform, however, was present in the blood to produce light anesthesia. The first part of the experiment indicates that the animal was more than normally resistant to chloroform poisoning, and this initial anesthesia may have still further raised the animal's resistance or natural immunity. The experiment supports the work of Matthews as to the rapidity of production of fibrinogen by the normal liver.

Dog 91.

February 22. A lean, female pup; weight 27 lbs. The animal was bled, under ether anesthesia, from the jugular vein. The fibrinogen was 0.7488 gm. per 100 c.c.

February 24. Chloroform anesthesia for two hours; ⅝ oz. given.

February 25. Animal seemed well and did not vomit.

February 26. The dog was bled from the jugular vein under ether anesthesia. The fibrinogen was 0.4404 gm. per 100 c.c. Twenty-five cubic centimeters of blood were analyzed for calcium. The calcium was 0.01248 gm. per 100 c.c.

March 3. The animal had a little distemper, but was fairly well.

March 19. The dog was bled from jugular vein under ether anesthesia. The fibrinogen was 0.3784 gm. per 100 c.c.

March 24. The animal appeared well and weighed 24 lbs.

April 7. Under ether anesthesia the common bile duct was ligated.

April 8. The dog seemed pretty well. Urine contained bile pigment.

April 11. Jaundice had developed as usual; the urine was very highly colored; and the stools were pasty and free from bile.

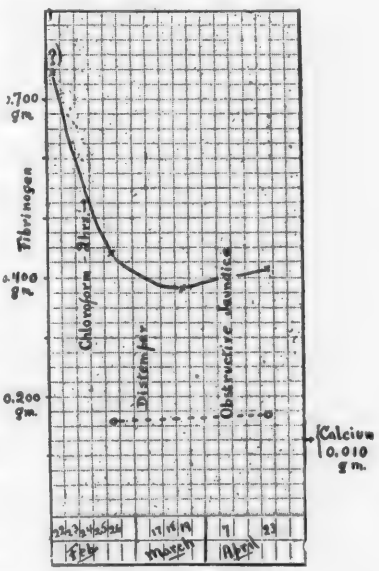

Chart 8. Dog 91.

April 20. The picture was typical of obstructive jaundice.

April 23. The animal weighed 21 lbs. All the tissues were deeply jaundiced. Under ether anesthesia the dog was bled from the carotids. The calcium was 0.0133 gm. per 100 c.c. and the fibrinogen was 0.4158 gm. per 100 c.c. The blood clotted in a normal manner. The clots were very tough and firm.

Autopsy.—The peritoneal cavity was normal except for old adhesions about the site of operation on the common bile duct. The tissues were everywhere deeply bile-stained. The heart, lungs, spleen, pancreas, stomach, and intestines were normal. There was a small punched out ulcer in the duodenum measuring 1 cm. in diameter. The gall bladder and the common cystic and hepatic ducts were all enormously dilated and a little thickened. They contained fluid bile. All the body fluids contained bile pigments.

Liver.—In the microscopical sections the liver cells in the central portion of each lobule showed some large and small fat droplets and contained, in addition, some greenish yellow granular pigment. There was an increase in wandering mononuclear cells about the tissue in the very centers of the lobules, but the greater part of all the lobules was practically normal; the cells had a normal appearance, and there was scarcely any evidence of the injury presumably caused by the chloroform anesthesia.

The curve of fibrinogen in this experiment (Chart 8) is of considerable interest. Possibly the first determination is too high, as this is one of the earliest, and was made before the value of centrifugalizing the precipitate was appreciated. The chloroform anesthesia was very well borne and probably caused only a slight drop in the fibrinogen. The determination of March 19 showed a slight diminution, and may possibly be explained by the poor condition of the animal, the result of distemper. The total obstructive jaundice, which lasted for a period of two weeks, caused no drop in the fibrinogen, but rather a slight increase. The calcium content of the blood likewise increased slightly.

TABLE VI.

Normal Dogs. Fibrinogen in 100 c.c. of blood.

March 3.	Dog 95.....	0.3820 gm.	2 separate fractions.
March 3.	Dog 95.....	0.3864 gm.	
March 15.	Dog 93.....	0.5348 gm.	
March 5.	Dog 97.....	0.5064 gm.	
June 6.	Dog 98.....	0.3572 gm.	

A review of Table VI and some of the other tables indicates that a normal dog may have a fibrinogen content averaging from 0.300 to 0.600 grams per 100 cubic centimeters of blood. Under fixed conditions an animal may have a pretty constant fibrinogen content and this may be half or double that of another healthy animal. We are not prepared to explain these differences, which we feel sure are not due to errors in the determinations. Perhaps some satis-

factory explanation may be reached when more evidence has been accumulated.

It may be worth while to say a few words about the parallelism between the liver necrosis and the drop in blood fibrinogen. Probably the injury to the liver is accomplished during the chloroform anesthesia, for after the anesthesia one is quite unable to protect an animal against the liver necrosis by bleeding, infusions, etc. The necrosis does not increase in extent during the second twenty-four hours, yet there is a rapid drop in fibrinogen, which usually reaches its minimum on the second day and may remain very low during the next two days. The hyaline appearance of the dead liver cells—a typical hyaline coagulative necrosis—is most striking during the second day.

Why does the blood fibrinogen drop in this manner or why does it not drop immediately after the chloroform anesthesia? Liver extirpation causes the fibrinogen to disappear in two hours. Nolf considers that it is anchored by the vaso- and leucothrombins which normally are held in check by the hepato-thrombin. Pawlow circulated the blood through the head, heart, and lungs, and observed that the blood would not clot after a few hours. One possibility is that only a part of the liver cells are injured and the rest are able to form some fibrinogen. But this does not explain the very low blood fibrinogen during the second and third days, when the necrosis is stationary and the repair has commenced in the uninjured liver cells and is moving ahead rapidly. It may be argued that the coagulative liver necrosis contains fibrin and uses up a good deal of the blood fibrinogen. Again, it is possible that the acute inflammatory reaction, which is at its height on the second and third days, and is naturally accompanied by much edema and swelling of the tissues, causes sufficient compression of the intact liver cells to embarrass their action.

We have been unable to discover a convincing argument to explain the rapid disappearance of the blood fibrinogen, although some of the factors mentioned above may explain it in part. This problem is bound up with that of the life history and general utility of the fibrinogen. It is hard to believe that the coagulation of blood is the only function of the blood fibrinogen. Is it not possible

that the blood fibrinogen is taken up by the various organs to replace their tissue waste? If it can be shown that fibrinogen is available as a tissue building proteid, then it is easy to explain all the phenomena under discussion. When all of the fibrinogen is removed by repeated blood defibrination (Chart 7), it was restored in twenty-four hours to more than half the normal, thus indicating the great reserve power of the liver. When the liver has actively regenerated following a severe chloroform poisoning, we find a rise of fibrinogen much above normal. This seems to indicate that the production of fibrinogen is an essential part of liver cell activity.

SUMMARY.

1. Chloroform anesthesia for two hours or more will cause more or less central liver necrosis in dogs, depending on the length of the anesthesia and the susceptibility of the animal.

2. If the fibrinogen of the blood of such an animal be estimated at intervals, it is found that this proteid shows a drop corresponding to the amount of liver necrosis.

3. By administering chloroform, the fibrinogen may be almost eliminated from the circulating blood, and the poisoned animal may bleed for hours from small skin pricks or cuts.

4. The liver can recover from a grave injury due to chloroform and return practically to a normal condition in about ten days.

5. The fibrinogen reappears in the blood as the liver effects its repair. It seems that the quantity of fibrinogen present is a good indicator of the liver efficiency and a fairly accurate index of the amount of liver injury.

6. Shortly after the recovery of the liver from an injury due to chloroform, one may find an excess of fibrinogen in the blood.

7. In severe cases of chloroform poisoning, the calcium of the blood was normal or slightly increased, and the thrombin was normal.

8. These experiments give no evidence that the formation of thrombin or prothrombin is dependent upon liver activity.

9. The hemorrhages of chloroform poisoning are due not to lack of blood clotting but to inefficient coagulation. The clot has not the body and toughness supplied by the fibrinogen, and is, therefore, unable to check even capillary hemorrhage.

10. Fibrinogen is either formed in the liver or is wholly dependent upon liver activity for its production.

In conclusion we desire to express our appreciation to Dr. W. H. Howell for his stimulating interest and invaluable assistance, and to Dr. W. W. Duke for observations on the formed elements of the blood.

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CHANGES IN THE PROTEOLYTIC ENZYMES AND ANTI-ENZYMES OF THE BLOOD SERUM PRODUCED BY SUBSTANCES (CHLOROFORM AND PHOSPHORUS) WHICH CAUSE DEGENERATIVE CHANGES IN THE LIVER.[1]

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The observations of Salkowski,[2] which have shown that incubated liver undergoes "self-digestion," have been extended to almost all tissues of the animal body. It is well known that the autolysis of most tissues occurs with maximum activity in a weakly acid medium. Biondi[3] has found that disintegration of liver is hastened by addition of weak acid, and Hedin and Rowland,[4] using the expressed juice of various organs, have noted that spleen, kidney, and lymphatic nodes autolyze more actively in acid than in neutral or alkaline media. The trivial autolysis of voluntary muscle is not materially affected by acid or alkali, but the autolysis of heart muscle resembles that of other organs. Levene and Stookey[5] have found that autolysis of brain tissue and of testicle is favored by the presence of acid.

The pancreas is not an exception to the foregoing rule. The well-known observations of Heidenhain[6] have shown that trypsin, which exhibits maximum activity in alkali, exists in the pancreas as inactive zymogen, so that fresh extracts of the organ cause little proteolysis under conditions which favor the activity of trypsin.

[1] Received for publication, November 4, 1910.

[2] Salkowski, *Ztschr. f. klin. Med.*, 1890, xvii, Suppl., p. 77.

[3] Biondi, *Virchows Arch. f. path. Anat.*, 1896, cxliv, 373.

[4] Hedin and Rowland, *Ztschr. f. physiol. Chem.*, 1901, xxxii, 531.

[5] Levene and Stookey, *Jour. Med. Research*, 1903, x, 212.

[6] Heidenhain, *Arch. f. d. ges. Physiol.*, 1875, x, 557.

The conditions under which acid activates pancreatic extracts and their relation to the activation of pancreatic juice by enterokinase have been studied by one of us (Dochez).[7] Fresh pancreatic tissue which undergoes active autolysis in the presence of weak acid, suffers little change in neutral solutions and is almost unchanged after incubation in an alkaline medium, the concentration of which agrees with that favorable to the action of trypsin (0.2 to 0.4 per cent. sodium carbonate). Heidenhain has shown that acid converts tryptic zymogen of fresh pancreas into trypsin; pancreas subjected to the action of acid acquires the power to digest under conditions which are favorable to trypsin, namely, in neutral or alkaline media. Although splenic substance autolyzes with greater activity in acid than in alkali, Hedin[8] has obtained in relatively purified form two enzymes, one of which, designated α-lieno-protease, acts in alkali, whereas the other, designated β-lieno-protease, acts in acid. If fresh spleen (Hedin[9]) is temporarily subjected to the action of weak acid, its ability to autolyze in the presence of alkali is materially increased. Pretreatment with acid makes active an enzyme which digests in the presence of alkali.

Dochez[10] has studied the modifications of hepatic autolysis which occur as the result of changes in reaction, and he has compared autolysis of liver with autolysis of fresh pancreas. Whereas fresh liver autolyzes with greater activity in acid (0.2 per cent. acetic acid) than in neutral or alkaline media (from 0.2 to 0.4 per cent. sodium carbonate), liver which has been treated with weak acid during twenty-four hours acquires the power to autolyze or to digest casein in alkaline medium with equal or greater activity than in acid. Other evidence cited by Dochez shows that the liver contains two enzymes, one of which acts in acid, whereas the other, which acts in alkali, is active only after the tissue has been subjected to acid. Vernon[11] has shown that pancreatic extracts become active after standing; Dochez has likewise noted that liver tissue allowed to stand on ice, gradually acquires the power to autolyze in the presence of alkali.

[7] Dochez, *Proc. Soc. Exper. Biol. and Med.*, 1910, vii, 97.
[8] Hedin, *Jour. Physiol.*, 1904, xxx, 155.
[9] Hedin, Festschrift für Olof Hammarsten, Upsala, 1906, vi, 1.
[10] Dochez, *Jour. Exper. Med.*, 1910, xii, 666.
[11] Vernon, *Jour. Physiol.*, 1901, xxvii, 269.

It is noteworthy that the polynuclear leucocytes of an inflammatory exudate cause proteolysis with greater activity in alkali than in acid and, unlike pancreas, liver or spleen and, doubtless, other organs, exhibit this activity in alkali when freshly obtained from the body; no pretreatment with acid is necessary in order to bring this enzyme (leucoprotease) into action. The bone marrow, unlike other tissues, exhibits the same ability to digest with greater activity in alkali than in acid.

The power of the blood serum to restrain the activity of tryptic digestion has long been known. Hahn[12] made the observation that trypsin fails to digest fibrin or gelatin when fresh blood serum is present. He found that this power to inhibit is lost if blood serum is heated to a temperature of 65° C. The inhibiting substance is apparently attached to the proteins of the serum, but is not a common property of all proteins of the blood, for the globulin fraction of the serum, precipitated by half saturation with ammonium sulphate, fails to restrain the action of trypsin, whereas the albumin fraction precipitated by complete saturation after globulin has been removed exerts a retarding influence.

Ascoli and Bezzola,[13] in 1903, doubtless influenced by the observations of Fr. Müller[14] upon self-digestion of lung consolidated by pneumonia, have sought to determine if this inhibiting property of the serum undergoes any alteration during the progress of pneumonia. They have claimed that there is at first a marked increase of anti-tryptic action; this increase is maintained for a time, and after crisis it is followed by a decrease, often occurring in association with disappearance of the local lesion. The subject attracted little attention until Brieger and Trebing[15] showed that there is an almost constant increase of the anti-tryptic activity of the blood serum in association with carcinoma. Subsequent studies have demonstrated that this increased anti-enzymotic activity is apparently dependent upon the accompanying cachexia rather than upon the new growth itself, and a similar change has been observed in association with a variety of diseases, such as pneumonia, typhoid

[12] Hahn, *Berl. klin. Wchnschr.*, 1897, xxxiv, 499.
[13] Ascoli and Bezzola, *Berl. klin. Wchnschr.*, 1903, xl, 391.
[14] Fr. Müller, *Verhandl. d. 20 Cong. f. inn. Med.*, 1902, 192.
[15] Brieger and Trebing, *Berl. klin. Wchnschr.*, 1908, lxv, 1041.

fever, tuberculosis, and exophthalmic goitre, of which the common characteristic, according to K. Meyer,[16] is increased disintegration of the protein constituents of the body, perhaps referable to increased activity of proteolytic enzymes.

Finding that the serum in certain diseases associated with leucocytosis exhibits increased anti-enzymotic activity when tested with enzyme of polynuclear leucocytes, several observers (Bittorf,[17] Wiens[18]) have maintained that the enzyme set free by leucocytes neutralizes the anti-body of the serum and thus indirectly causes an excessive regeneration. Nevertheless, numerous observations have shown, on the one hand, that anti-enzymotic activity of the blood serum is not constantly increased in association with leucocytosis (K. Meyer), whereas, on the other hand, many conditions, such as malignant growth, tuberculosis, and typhoid fever, frequently accompanied by increase of anti-enzyme, usually exhibit no leucocytosis.

By a series of comparative tests, Jochmann and Kantorowicz[19] have shown that serum which exhibits high anti-enzymotic activity toward trypsin shows increased ability to restrain the activity of the enzyme of the polynuclear leucocytes, which like trypsin digests in the presence of an alkaline medium. Injection of trypsin into rabbits increases the ability of the serum to inhibit both trypsin and leucoprotease, and, furthermore, injection of leucoprotease has the same result.

The occurrence of increased anti-enzyme in the serum suggests the possibility that anti-enzyme is increased in order to balance an increased quantity of free enzyme. With this possibility in view, we have attempted to determine if the proteolytic activity of the blood serum undergoes any alteration during the course of chloroform poisoning so severe that advanced degenerative changes are produced in the liver. The possibility that such a study might prove fruitful has been suggested by the observation that increase of anti-leucoprotease accompanies prolonged intoxication with chloroform or phosphorus.

[16] K. Meyer, *Berl. klin. Wchnschr.*, 1909, xlvi, 1064, 1890.
[17] Bittorf, *Deutsch. Arch. f. klin. Med.*, 1907, xci, 212.
[18] Wiens, *Deutsch. Arch. f. klin. Med.*, 1907, xci, 456.
[19] Jochmann and Kantorowicz, *Ztschr. f. klin. Med.*, 1908, lxvi, 153.

The existence of proteolytic enzyme in normal blood serum has long been known. Delezenne and Pozerski[20] have found that blood serum mixed with chloroform acquires the power to digest gelatin and casein. Hedin has shown that the globulin fraction of the blood serum of the ox, obtained by half saturation with ammonium sulphate, contains a weak proteolytic enzyme. The albumin fraction of the serum obtained by complete saturation after removal of globulin contains anti-enzyme, which in normal serum restrains the activity of the enzyme just mentioned. One of us[21] has shown that the anti-enzymotic activity of the blood serum is lost in the presence of 0.2 per cent. acetic acid; in this medium the blood serum exhibits well marked proteolytic activity, but in neutral or alkaline media anti-body balances enzyme, and neither autolysis nor other proteolytic activity is demonstrable.

In order to determine what factors cause changes in the enzymotic and anti-enzymotic activity of the blood serum, a condition which can be produced experimentally offers obvious advantages, for the phenomena which occur can be repeatedly traced from the beginning through various stages of the process. Chloroform poisoning has proved especially favorable for this study because it reproduces the increase of anti-enzyme heretofore frequently noted with the cachexia of carcinoma, with various infections, and with other diseases.

The changes in the liver which accompany prolonged administration of chloroform by inhalation, are clearly defined by the recent studies of Howland and Richards[22] which have directed our attention to this subject. There is necrosis implicating the central part of each hepatic lobule. Disintegration of protein within the body is shown by increased elimination of nitrogen and sulphur by the urine. When intoxication is not severe, there is, instead of necrosis, fatty degeneration of the liver, kidneys, heart muscle, etc. Doyon and Billet[23] have found that the blood may become incoagulable and that incoagulability is associated with diminution of the fibrinogen content of the serum.

[20] Delezenne and Pozerski, *Compt. rend. Soc. de biol.*, 1903, lv, 327, 690, 693.
[21] Opie, *Jour. Exper. Med.*, 1905, vii, 316.
[22] Howland and Richards, *Jour. Exper. Med.*, 1909, xi, 344.
[23] Doyon and Billet, *Compt. rend. Soc. de biol.*, 1905, lviii, 852.

We have followed the coagulability of the blood during the course of chloroform poisoning produced by the daily administration by stomach of a given quantity of chloroform (one or two cubic centimeters per kilogram of body weight), and have found that the coagulation time of the blood increases during the first three days; in some instances the blood becomes wholly incoagulable. Hemorrhage into the gastro-intestinal tract or into the peritoneal cavity is not infrequent, and death often occurs at the end of three or four days. When poisoning is not acutely fatal, a reaction occurs; the coagulability of the blood rapidly regains its former activity, and even though the same daily dose of chloroform is administered, coagulation at the end of about a week after the beginning of the experiment may have become somewhat more rapid than normal. An animal which has survived the critical period of three or four days may live ten or twelve days. The animal has established some form of resistance to the poison. In such instances there is widespread fatty degeneration of the liver and other organs and comparatively little necrosis.

The phenomena which have been described have a close analogy in phosphorus poisoning. There is, it is well known, intense fatty degeneration of the liver with some necrosis, and, in some instances, as Jacoby[24] has shown, diminution of the coagulability of the blood is associated with disappearance of fibrinogen. Furthermore, Jacoby has found that the liver with phosphorus poisoning undergoes more rapid autolysis than normal liver. He suggests that enzyme set free by the liver dissolves the fibrinogen of the blood. Our observations, mentioned above, suggest that changes which occur in the blood as the effect of chloroform or phosphorus may be subject to considerable variation, dependent upon the severity of intoxication and upon the length of the period of administration.

Gradual increase of anti-enzyme for a proteolytic enzyme, namely, leucoprotease, during the progress of intoxication with chloroform and phosphorus, noted at the beginning of our experiments, has suggested the possibility that disintegrating liver tissue may set free those proteolytic enzymes which are demonstrable in normal liver. The attempt has been made to determine if at any stage of intoxi-

[24] Jacoby, *Ztschr. f. physiol. Chem.*, 1900, xxx, 174.

cation the serum exhibits increased proteolytic activity. The enzymotic content of the serum has been tested by incubating a fixed quantity of blood serum during four or five days at 37° C. and subsequently determining the degree of autolysis, or by incubating a fixed quantity of serum with a suitable protein substrate, such as heated blood serum. That hepatic enzyme, which is most readily demonstrated, digests in the presence of acid. To determine if the similar enzyme which exists in the blood undergoes any change during the progress of chloroform intoxication, a series of experiments has been performed on dogs.

A fixed quantity of blood has been subjected to autolysis in the presence of 0.2 per cent. acetic acid. Blood has been drawn at intervals of three or four days by means of a needle inserted through the skin into the jugular vein. A measured amount of serum obtained by centrifugalization of the whipped blood has been diluted with salt solution (0.85 per cent.) and acetic acid in such proportion that the final volume of twenty-five cubic centimeters contains 0.2 per cent. acetic acid. In some instances blood serum denaturalized by heat has been added to the mixture as substrate for the proteolytic enzyme of the serum. All mixtures have been incubated during four or five days at 37° C. A mixture containing the same ingredients has served as control and has been immediately heated to boiling. Since it is undesirable to withdraw repeatedly considerable quantities of blood, the quantity of serum which has been subjected to autolysis has been small and occasionally, for lack of material (*e. g.*, Experiment 9), it has been necessary to omit this control. After coagulation by heat, the coagulum has been removed by filtration, and nitrogen in the filtrate has been determined by the Kjeldahl method; the amount of nitrogen liberated by digestion of coagulable protein will be expressed in cubic centimeters of one-tenth normal sulphuric acid.

In some experiments autolytic activity of serum from an animal receiving daily doses of chloroform has been tested on different days; the first test, made before administration of chloroform, serves as a normal standard for comparison with subsequent changes. In other instances the serum of animals which have received chloroform has been compared with normal serum from another animal.

Repeated tests, some of which will be recorded, have demonstrated that autolysis in neutral or alkaline media under conditions otherwise similar to those just described fails to occur or is much less marked than autolysis in the presence of acid.

EXPERIMENT 1.—A dog (weight 6,950 grm.) received on two successive days 10 c.c. of chloroform. Coagulation time of the blood before administration of chloroform was four minutes and five seconds. On the third day of the experiment the animal was sick and the blood failed to coagulate within one hour. The following figures represent autolysis in the presence of acid before and after administration of chloroform. Furthermore, serum obtained on the third day has been compared with the serum of a normal dog.

	Autolysis of 5 c.c. of serum during 4 days at 37°.			
	Control.	In neutral medium.	0.2 per cent. sodium carbonate.	0.2 per cent. acetic acid.
Serum of chloroform dog before administration of chloroform. (First day; normal serum.).....	1.35			3.0
Serum of chloroform dog on third day.....	1.5	1.7	2.5	6.45
Serum of normal dog.....	1.75	1.75	2.05	3.6

The accuracy of the figures obtained with serum of the dog which had received chloroform (3d day) and normal serum, autolyzed in the presence of acid, is confirmed by an additional test made with 3 c.c. of each serum tested in the same medium: serum of chloroform dog (3d day), 4.3; normal serum, 2.8.

The administration of chloroform has caused well marked increase in the autolytic activity of blood serum tested in acid, and little change when tested in alkaline (perhaps trivial increase) or in neutral medium.

Since the quantities subjected to autolysis are small, experiments have been multiplied with the purpose of establishing the accuracy of the result. In Experiment 2, in addition to the tests previously employed, serum and protein substrate (denaturalized serum) have been incubated in the presence of acetic acid.

EXPERIMENT 2.—A dog (weight 6,750 grm.) received on four successive days 10 c.c. of chloroform. Blood was drawn on the first day (coagulation time three minutes, forty seconds), on the fourth day (five minutes, twenty-one seconds), and on the fifth day (four minutes, twenty-three seconds).

	Autolysis of 3 c.c. of serum during 4 days at 37°.			1 c.c. serum, substrate, and 0.2 per cent. acetic acid.
	Control.	In neutral medium.	0.2 per cent. acetic acid.	
1st day (normal)	1.0	1.05	3.7	2.85
4th day	1.1	1.05	5.05	3.45
5th day	1.8	1.75	4.95	4.15

EXPERIMENT 3.—A dog received chloroform by mouth on fourteen successive days. The serum was tested as follows:

	Autolysis of 3 c.c. of serum during 5 days at 37°.		
	Control.	In neutral medium.	0.2 per cent. acetic acid.
1st day (normal)	1.2	—	2.4
14th day	—*	1.9	5.65

* Control on fourteenth day doubtless approximates closely digestion in neutral medium (1.9 c.c.) and certainly does not exceed this figure.

EXPERIMENT 4.—A dog (weight 7,000 grm.) received on four successive days 10 c.c. of chloroform. Blood was drawn on the first day (coagulation time, four minutes), on the fourth day (nine minutes, twenty seconds), and on the fifth day (six minutes, twenty-one seconds). The animal was moribund on the fifth day.

	Autolysis of 3 c.c. serum during 5 days at 37°.			1 c.c. serum, substrate, and 0.2 per cent. acetic acid.
	Control.	In neutral medium.	0.2 per cent. acetic acid.	
1st day (normal)	[1.05]*	1.05	3.2	2.65
4th day	2.1	1.9	4.6	4.3
5th day	5.85	5.9	9.2	8.15

* This control was not determined, as the available serum was insufficient; other experiments show that normal serum incubated in neutral medium gives the same figure as serum coagulated immediately. It has been assumed that this control is identical with the figure obtained after incubation with reaction unchanged.

In Experiment 4, increase of the control is noteworthy; the serum contains, in greatly increased quantity, nitrogenous substances incoagulable by heat. Nevertheless, when these controls are subtracted from the figures obtained after incubation, evidence of increased proteolytic activity is obtained.

In the following experiments, which confirm those recorded

above, there is moderate increase of proteolytic activity, which reaches a maximum on the fourth day of chloroform intoxication.

EXPERIMENT 5.—A dog (weight 5,000 grm.) received on six successive days 10 c.c. of chloroform. Blood was drawn on the first day (coagulation time, one minute, twenty-seven seconds), on the fourth day (coagulation time, eight minutes, fifteen seconds), and on the seventh day (coagulation time, six minutes, six seconds).

	Autolysis of 3 c.c. serum during 4 days at 37°.			3 c.c. serum, substrate, and 0.2 per cent. acetic acid.
	Control.	In neutral medium.	0.2 per cent. acetic acid.	
1st day (normal)	0.9	1.05	2.8	2.55
4th day	0.9	1.0	3.7	2.7
7th day	1.0	1.05	3.7	2.85

EXPERIMENT 6.—A dog (weight 4,750 grm.) received on ten out of eleven days (sixth day excepted) 7 c.c. of chloroform. Coagulation time: first day, five minutes, eighteen seconds; fourth day, four minutes, thirty-four seconds; seventh day, two minutes, seven seconds; tenth day, three minutes, fifteen seconds.

	Autolysis of 4 c.c. serum during 4 days at 37°.	
	Control.	0.2 per cent. acetic acid.
1st day (normal)	1.2	3.15
4th day	1.2	3.95
7th day	1.5	3.75
10th day	1.5	4.5

EXPERIMENT 7.—A dog (weight 5,600 grm.) received on six out of seven days (none on sixth day) 7 c.c. of chloroform. Coagulation time: first day, five minutes, thirty-one seconds; fourth day, seven minutes, seventeen seconds; seventh day, seven minutes, fifteen seconds.

	Autolysis of 4 c.c. serum during 5 days at 37°.	
	Control.	0.2 per cent. acetic acid.
1st day (normal)	1.2	3.45
4th day	1.45	4.85
7th day	1.55	4.35

The experiments demonstrate that the well marked increase of proteolytic activity exhibited by animals which have received chloroform, attains its maximum on the third or fourth day of intoxication, and tends to be greatest when intoxication is most severe and when delay of coagulation time is considerable. Increased activity of autolysis may be absent when the critical period of intoxication

has passed or when the injurious action of chloroform has been slight. In the following experiment there has been no evidence of increased enzymotic activity.

EXPERIMENT 8.—A dog received 10 c.c. of chloroform on six successive days. Coagulation time: first day, three minutes, forty-five seconds; fourth day, ten minutes, sixteen seconds; seventh day, two minutes, twelve seconds.

	Autolysis of 3 c.c. serum during 4 days at 37°.			3 c.c. serum, substrate, and 0.2 per cent. acetic acid.
	Control.	In neutral medium.	0.2 per cent. acetic acid.	
1st day (normal)	1.0	1.1	4.0	2.85
4th day	1.15	1.5	4.0	3.0
7th day	2.5	2.4	3.9	3.8

The very active anti-enzymotic activity of this serum will be mentioned later.

In the following experiment there is a close relationship between proteolytic activity and severity of intoxication, well indicated by delay of coagulation time.

EXPERIMENT 9.—A small quantity of serum (1 c.c.) has been allowed to act upon coagulated protein (5 c.c. of heated blood serum) during five days at 37° C. The sera of four dogs which have received 7 c.c. of chloroform on two successive days have been drawn on the third day, and the proteolytic activity in neutral and acid media compared with that of the sera from two normal dogs.

	Coagulation time.		Serum + heated serum after 5 days at 37°.	Serum + heated serum + 0.2 per cent. acetic acid after 5 days at 37°.
	Minutes.	Seconds.		
Normal Dog A	1	15	1.7	1.65
Normal Dog B	2	30	1.75	1.75
Chloroform Dog A	7	30	1.85	2.1
Chloroform Dog B	9	30	1.65	2.15
Chloroform Dog C	16	30	2.35	2.25
Chloroform Dog D	30	0	2.65	3.55

On the fourth day Chloroform Dog C was much less sick than on the previous day, whereas Chloroform Dog D was moribund. The following data were obtained:

	Coagulation time.		Serum + heated serum after 5 days at 37°.	Serum + heated serum + 0.2 per cent. acetic acid after 5 days at 37°.
	Minutes.	Seconds.		
Chloroform Dog C	12	30	1.6	2.3
Chloroform Dog D	21	0	2.8	4.45

The figures obtained are small, but the changes which occur are the same as those previously described. It is noteworthy that the coagulation time of the blood is a fair index of the intoxication caused by chloroform. There has been a close relationship between coagulation time and autolytic activity, both in an acid and in an approximately neutral medium. The close relation between the toxic effect of chloroform and autolytic activity of the blood serum is well illustrated by the tests made on the fourth day. Dog C has in part recovered from the depression produced by the first two doses, and its serum exhibits autolytic activity not far removed from normal, whereas the serum of Dog D, which has been profoundly poisoned, exhibits increased autolytic activity.

Having in view the possibility that degenerative changes in the liver are associated with increased activity of proteolytic enzymes, we have undertaken a series of experiments to determine if the blood serum of animals receiving chloroform or phosphorus exhibits any alteration of the normal power of blood serum to restrain the autolysis of liver.

EXPERIMENT 10.—A dog has been given daily doses of chloroform (2 grm. per kilo of body weight) during ten days. An emulsion has been prepared by mixing finely ground liver with four times its volume of salt solution; 20 c.c. of this mixture have been allowed to undergo autolysis during five days at 37° C. both alone and in the presence of blood serum from the same animal and from a normal animal. The volume of the mixture has been increased in every instance to 25 c.c. Autolysis of liver alone is represented by 25.7 c.c. N/10 sulphuric acid.

	Normal serum.	Chloroform serum.
Liver + 1 c.c. serum	22.25	20.2
Liver + 2.5 c.c. serum	18.4	18.65
Liver + 5 c.c. serum	15.5	17.05

Blood serum has inhibited autolysis in slight degree, but no noteworthy change of inhibition has been caused by administration of chloroform. When a small quantity of serum has been employed, inhibition has been slightly greater with chloroform than with normal serum. It is probable that the slightly higher figure obtained with five cubic centimeters of serum of the animal which received chloroform, is referable to its greater content of nitrogenous substances incoagulable by heat.

In other experiments, fresh liver of the rabbit has been subjected to autolysis in the presence of serum of a normal rabbit and of serum of a rabbit poisoned with phosphorus.

EXPERIMENT 11.—A suspension of normal rabbit's liver (10 c.c.) has undergone autolysis represented by 5.95 c.c. N/10 sulphuric acid (control, 2.05 c.c.). The following figures represent the effect of normal serum and of serum of a rabbit receiving phosphorus upon the same quantity of this suspension:

	Normal serum.	Chloroform serum.
Liver + 0.25 c.c.	6.1	6.0
Liver + 0.5 c.c.	5.45	6.05
Liver + 1 c.c.	5.65	5.1

There has been no constant or noteworthy difference in the effect of normal serum and of serum after administration of phosphorus upon autolysis of normal liver.

In a subsequent experiment the liver of an animal which has received phosphorus has been allowed to undergo autolysis. The attempt has been made to determine if serum of an animal similarly treated with phosphorus differs from normal serum in its effect upon the autolysis of phosphorus liver.

EXPERIMENT 12.—One gram of liver exhibiting fatty degeneration as the result of phosphorus has been suspended in 25 c.c. of salt solution and allowed to autolyze alone and in the presence of serum.

	Control.	After incubation during 5 days at 37°.	Digestion.
Liver	2.6	6.05	3.45
Liver + 2 c.c normal serum	3.1	5.05	1.95
Liver + 2 c.c. phosphorus serum	3.7	6.45	2.75

Both normal and phosphorus serum have inhibited autolysis of the liver of an animal which has received phosphorus. Inhibition has been less with the serum obtained after administration of phosphorus than with that of the normal animal, but the foregoing experiments offer little evidence that the anti-enzymotic activity of the phosphorus animals undergoes any significant change.

Far more constant results have been obtained when the serum of animals which have received chloroform has been allowed to act upon an enzyme which, unlike the autolytic enzyme of the liver,

acts with greatest activity in an alkaline medium. Various quantities of serum from animals which have received chloroform and of serum from normal animals have been allowed to act upon a fixed quantity of enzyme of polynuclear leucocytes (leucoprotease).

Leucoprotease (twenty milligrams) has been allowed to digest coagulated blood serum both alone and in the presence of normal and of chloroform serum.

EXPERIMENT 13.—The serum of a dog which has received 160 c.c. of chloroform during fourteen days has been employed.

	Control.	After incubation during 5 days at 37°.
20 mgr. leucoprotease + coagulated serum	1.55	19.4
	Normal serum.	Chloroform serum
Above mixture + 0.25 c.c. serum	19.25	12.1
Above mixture + 0.5 c.c. serum	15.55	4.35
Above mixture + 1.0 c.c. serum	5.45	3.3

Inhibition has been uniformly greater with the serum of the animal which has received chloroform than with the serum of a normal animal.

The experiment has been repeated with serum of a dog which has received repeated doses of chloroform administered by mouth.

EXPERIMENT 14.—

	Control.	After incubation during 5 days at 37°.
20 mgr. leucoprotease + coagulated serum	1.9	20.35
	Normal serum.	Chloroform serum.
Above mixture + 0.25 c.c. serum	20.3	15.4
Above mixture + 0.5 c.c. serum	19.1	4.0
Above mixture + 1.0 c.c. serum	8.8	3.2

Small quantities (0.2 and 0.5 cubic centimeter) of normal serum produce almost no effect upon the enzyme, yet the same quantities of chloroform serum greatly diminish its activity.

Continued increase of anti-enzymotic activity of the blood serum is well seen in the following experiment in which tests repeated at intervals of three days have been made with the serum of an animal to which chloroform has been administered daily.

EXPERIMENT 15.—A dog weighing 4,750 grm. has received daily during eleven days (the sixth day excepted) 12 c.c. of chloroform. From 25 to 35 c.c. of

blood have been drawn at intervals of three days. Coagulation time: first day, five minutes, eighteen seconds; fourth day, four minutes, forty-three seconds; seventh day, two minutes, seven seconds; tenth day, three minutes, fifteen seconds. On the fifth day there has been slight jaundice which has subsequently increased in intensity. The inhibition of 20 mgr. leucoprotease caused by 0.5 c.c. of serum is shown by the following figures:

	After incubation during 5 days at 37°.
20 mgr. leucoprotease + coagulated blood serum	17.25
Same mixture + 0.5 c.c. serum on 1st day (normal)	13.75
Same mixture + 0.5 c.c. serum on 4th day	12.45
Same mixture + 0.5 c.c. serum on 7th day	5.25
Same mixture + 0.5 c.c. serum on 10th day	2.9

The following test shows that administration of phosphorus has the same effect as administration of chloroform upon the anti-enzymotic activity of the blood serum.

EXPERIMENT 16.—A dog weighing 7,700 grm. has received 1/50 grain of phosphorus every second day during fourteen days. Its blood serum (coagulation time, four minutes, nine seconds) has been compared with two normal sera.

	After incubation during 5 days at 37°.
20 mgr. leucoprotease + coagulated serum	17.25
Same mixture and 0.5 c.c. normal serum A	13.75
Same mixture and 0.5 c.c. normal serum B.	15.15
Same mixture and 0.5 c.c. phosphorus serum	7.3

One-half cubic centimeter of normal serum exhibits slight anti-enzymotic activity, whereas the same amount of serum from an animal which has received phosphorus causes strong inhibition of an equal amount of enzyme.

Increased inhibition of leucoprotease, demonstrable in the serum of dogs which have received chloroform or phosphorus, has been found in rabbits as well.

EXPERIMENT 17.—In the following series of tests, sera from a rabbit which has received phosphorus has been obtained on different days and has been compared with normal sera.

20 mgr. leucoprotease + coagulated blood serum: control, 2.8; after incubation during 5 days at 37° C., 19.45.

With serum of the animal which has received small doses of phosphorus, digestion caused by leucoprotease is constantly less than with normal sera.

		Normal serum.	Phosphorus serum.
A	Same mixture + 0.25 c.c. serum	15.2	10.75
	Same mixture + 0.5 c.c. serum	7.05	5.2
	The following test has been made 4 days later:		
B	Same mixture + 0.25 c.c. serum	16.95	10.9
	The following test has been made one day after last:		
C	Same mixture + 0.25 c.c. serum	16.55	11.95
	Same mixture + 0.5 c.c. serum	7.25	4.6

The foregoing experiments show that the blood serum under the influence of a poison such as chloroform, acting with intensity sufficient to cause necrosis of the liver and diminish the coagulability of the blood, acquires increased ability to cause digestion of protein. This increased proteolysis is, in part at least, referable to an enzyme which, like the autolytic enzyme of the liver and almost all other organs, digests with maximum activity in a weakly acid medium. It is not improbable that proteolytic enzyme is liberated by disintegration of parenchymatous cells and carried away by the blood.

In animals which survive the severe intoxication produced by large doses of chloroform, or in those which have repeatedly received smaller doses, the blood serum acquires increased ability to restrain the action of a proteolytic enzyme; but this anti-enzymotic action is exerted, not upon the enzyme of the liver, which acts in acid, but upon an enzyme which acts in the presence of alkali, namely, leucoprotease. There is little reason to doubt that the same serum will restrain the action of a second enzyme which acts in alkali, namely, trypsin, for, on the one hand, Jochmann and Kantorowicz have found that anti-enzyme for enzyme of leucocytes and for trypsin increases simultaneously, and, on the other hand, K. Meyer has found the anti-tryptic activity of the serum increased in animals which have received phosphorus. Anti-enzyme for one type of enzyme is increased; whereas evidence heretofore available indicates that a second type of enzyme is predominant in those tissues which are subject to the destructive action of the poison. Furthermore, our experiments have shown that an enzyme similar to that of the injured organ accumulates in the blood serum. It is difficult to correlate these observations.

Nevertheless, the experiments of Hedin and of Dochez show that

the liver contains a second enzyme which, unlike that directly obtainable from fresh liver, digests protein in the presence of an alkaline medium. These observations suggest the possibility that this enzyme may have a part in changes which increase the anti-enzymotic activity of the blood serum after administration of substances which injure the liver. Treatment of fresh liver with acid discloses an enzyme which digests in the presence of acid. Does the serum of an animal which has received repeated doses of chloroform and exhibits increased power to inhibit leucoprotease and, doubtless, trypsin, exhibit as well increased power to inhibit that enzyme of the liver which is similar to these two enzymes? The experiments which follow have been undertaken with the purpose of answering this question.

Using the method described by one of us (Dochez), we obtained that enzyme which digests protein in the presence of alkali, by subjecting fresh liver to the action of weak acetic acid during a period of twenty-four hours. Liver just after removal with aseptic precautions from an etherized animal has been finely ground by means of a hashing machine; ground liver has been mixed with twice its volume of salt solution to which acetic acid has been added in such amount that the concentration of the mixture is 0.2 per cent. After this mixture has stood at a temperature slightly above freezing during twenty-four hours, the acid has been neutralized by an equivalent quantity of one-tenth normal sodium hydroxide. Ten cubic centimeters of this neutralized mixture has been allowed to undergo autolysis and the inhibition caused by various quantities of serum has been tested. In order to determine if the inhibitory activity of serum is increased during the progress of chloroform poisoning, normal serum has been compared with serum obtained after repeated administration of chloroform. For the purpose of the present investigation, a comparative study of anti-enzymotic activity for leucoprotease and for enzyme of liver has been essential, and parallel tests of the action of each serum upon the two enzymes have been made.

The change which occurs rapidly when liver is treated with acid occurs slowly when liver in approximately neutral suspension is preserved under conditions which prevent bacterial growth. In the

following experiment a suspension allowed to stand under toluol during two months has been used.

EXPERIMENT 18.—Two dogs, Dogs A and B, have received daily 10 c.c. of chloroform by stomach. Blood has been drawn on the first and on the fourth day of the experiment. Anti-enzymotic activity of this serum has been tested by allowing a given quantity of leucoprotease (20 mgr.) to act upon substrate (5 c.c. heated blood serum) in the presence of 0.5 c.c. of blood serum obtained before administration of chloroform and on the fourth day of administration. A parallel test has been made with liver which has been allowed to stand on ice over toluol during two months in order to liberate that enzyme which, like leucoprotease, digests in alkali. This suspension of liver has been made by mixing equal volumes of liver, ground through a hashing machine, and of salt solution with sodium carbonate in the proportion of 0.1 per cent. Ten cubic centimeters of this suspension diluted with salt solution so that the total volume is 25 c.c. has been allowed to undergo autolysis; the same quantity has been autolyzed in the presence of blood serum.

Leucoprotease (20 mgr.) acting upon the substrate is represented by 16.2 c.c. N/10 H_2SO_4 (control, 1.8 c.c.). Autolysis of liver (10 c.c.) alone is represented by 7.5 c.c. N/10 H_2SO_4 (control 3.3 c.c.). The following table represents a comparison of digestion by leucoprotease and autolysis of liver in the presence of the same serum.

	Digestion with leucoprotease + 0.5 c.c. serum.	Autolysis of active liver.	
		+ 0.5 c.c. serum.	+ 1 c.c. serum.
CHLOROFORM DOG A.			
With 0.5 c.c. serum obtained before administration of chloroform ...	13.6	6.7*	8.15
With 0.5 c.c. serum obtained on 4th day of chloroform administration	3.35	5.2	—
CHLOROFORM DOG B.			
With 0.5 c.c. serum obtained before administration of chloroform ...	11.4	8.35	7.55
With 0.5 c.c. serum obtained on 4th day of chloroform administration	3.15	5.3	5.45

* This figure should be somewhat higher, since loss occurred as result of accident during Kjeldahl determination.

On the fifth day of the experiment the serum of each dog has been again tested, but as it has been necessary to use for autolysis a different suspension of liver, no comparison with the foregoing test is possible and consequently the two sera have been compared with sera from two normal dogs. Liver suspended in an equal volume of salt solution has been allowed to stand in the ice-chest under toluol during approximately two months; slight acidity of the suspension has been neutralized by addition of N/10 NaOH (4.8 c.c. to 170 c.c. of suspension). Ten cubic centimeters of the suspension have been used for each test; 5 c.c. of blood serum have been added with the purpose of supplying additional substrate for the proteolytic enzyme derived from the liver.

Digestion after incubation during five days at 37° C. is represented by 12.95 c.c. N/10 H_2SO_4 (control 4.8 c.c.).

	With 0.5 c.c. serum.	With 1 c.c. serum.
Serum of Chloroform Dog A	7.65	7.1
Serum of Chloroform Dog B	7.6	5.65
Serum of Normal Dog A	9.65	6.95
Serum of Normal Dog B	8.2	7.3

The degree of inhibition will obviously depend upon the strength of the enzyme and of the anti-enzymotic activity of the serum. In the second series of tests, one cubic centimeter of normal serum has so materially reduced the activity of the enzyme that differences between normal serum and serum after administration of chloroform are in large part obliterated.

In the first series of tests (Experiment 18), the inhibiting action of each serum upon leucoprotease has been compared with its action upon enzyme of liver; with well marked decrease of anti-enzyme for the one, there has been a parallel increase for the other. In the following experiment the serum tested has shown no noteworthy decrease of anti-enzyme for leucoprotease and there has been little, if any, change in its ability to restrain the similar enzyme of liver.

Experiment 19.—Two animals received on four successive days 10 c.c. of chloroform. Blood was drawn before administration and on the fourth day of the experiment. The effect of these sera upon leucoprotease and upon enzyme of liver is shown by the following figures:

Leucoprotease (20 mgr.) acting upon substrate is represented by 16.2 c.c. N/10 H_2SO_4 (control, 1.8 c.c.). Autolysis of liver alone is represented by 7.5 c.c. N/10 H_2SO_4 (control, 3.3 c.c.).

	Digestion with leucoprotease + 0.5 c.c. serum.	Autolysis of active liver + 0.5 c.c. serum.
Chloroform Dog C.		
With serum obtained before administration of chloroform	9.5	7.9
With serum obtained on 4th day after chloroform administration	8.5	7.55
Chloroform Dog D.		
With serum obtained before administration of chloroform	14.2	7.75
With serum obtained on 4th day of chloroform administration	13.7	7.85

In the following experiment serum of an animal which has received chloroform is compared with that of a normal dog.

EXPERIMENT 20.—A dog weighing 21 kilograms has received daily doses of chloroform increasing from 0.5 to 7 c.c. Blood has been drawn on the fourteenth day of the experiment. Inhibition of leucoprotease and of enzyme of liver are compared.

Leucoprotease (20 mgr.) acting on substrate causes proteolysis represented by 16.6 c.c. N/10 H_2SO_4, control being 1.5. Autolysis of the liver employed is represented by 27.2 c.c. N/10 H_2SO_4, control being 16.05 c.c. In order to make the degree of inhibition more conspicuous, the control has been subtracted from the figure obtained after digestion during four days at 37° C.

	Total digestion with leucoprotease + 0.5 c.c. serum.	Total autolysis of liver.	
		+ 0.5 c.c. serum.	+ 1 c.c. serum.
With serum of normal dog	14.9	12.7	12.15
With serum of chloroform dog	2.8	8.45	9.75

Well marked increase of anti-enzyme for liver pretreated with acid corresponds with increased anti-enzyme for leucoprotease. In the following experiment, identical in other respects with that just described, the effect upon autolysis of pretreated liver, both of normal serum and of serum from a dog which has received chloroform, has been tested in the presence of alkali.

EXPERIMENT 21.—The foregoing experiment has been repeated by comparing serum of a normal dog with serum of a dog (weighing 21 kilograms) which has received 21 c.c. of chloroform daily (the seventh day excepted) during ten days.

The leucoprotease and suspension of liver used in Experiment 20 have been employed and the control has been subtracted from the figure obtained after digestion during four days at 37° C.

	Total digestion with leucoprotease + 0.5 c c. serum.	Total autolysis of liver.	
		+ 0.5 c,c. serum.	+ 1 c.c. serum.
With serum of normal dog	13.2	15.65	13.8
With serum of chloroform dog	4.67	12.9	12.85

In order to obtain further information concerning the progress of the changes which effect the anti-enzymotic activity of the blood serum, comparative tests have been made with leucoprotease and with pretreated liver during the course of prolonged administration of chloroform. The method of testing inhibition is identical with

that previously employed; no alkali has been added to the mixtures prepared for proteolysis.

EXPERIMENT 22.—Serum has been obtained from a dog weighing 9 kilograms, which during nineteen days has received 9 c.c. of chloroform daily. Coagulation time, which has been two minutes and ten seconds before administration of chloroform, has been tested whenever blood has been drawn and has never risen above three minutes and thirty-five seconds. The animal has been jaundiced on the eighteenth day of the experiment.

The following table gives the results of a large series of tests made on different days with various quantities of serum added to mixtures containing either leucoprotease or liver rendered active by pretreatment with acid.

	Proteolysis with leucoprotease.			Autolysis of pretreated liver.				
	With no serum.	0.25 c.c. serum.	0.5 c.c. serum.	With no serum.	0.25 c.c. serum.	0.5 c.c. serum.	1 c.c. serum.	2 c.c. serum.
1st day (normal) .	12.9	11.65	9.5	16.2	12.5	11.2	10.5	8.9
4th day.....		7.1	4.45	15.0	9.8	8.45	7.55	6.9
7th day.....		6.5	4.4	18.2	10.1	9.0	8.05	8.0
10th day.....		6.7	5.4	16.6	9.9	9.5	7.6	7.1
14th day.....	15.7	6.2	4.6	16.3	10.5	10.0	8.8	8.1
18th day.....	14.8	4.4	3.4	15.9	9.3	8.5	7.1	7.0

Although there are some irregularities in the progress of the changes represented by the foregoing figures, it is obvious that the power of the serum to inhibit the active enzyme of acid-treated liver exhibits an increase parallel with the increased power to inhibit leucoprotease. Here, as in previous experiments, larger quantities even of normal serum (one or two cubic centimeters) cause such well marked inhibition of the hepatic enzyme that differences are less conspicuous than when small quantities (0.25 or 0.5 cubic centimeter) are employed. To demonstrate more clearly the parallel increase of anti-enzyme for the two enzymes which have been compared, curves (Fig. 1.) have been plotted to represent digestion caused by hepatic enzyme (unbroken line) and leucoprotease (broken line) in the presence of 0.25 and of 0.5 cubic centimeter of blood serum.

The experiments which have been described show that chloroform given in quantity sufficient to produce profound intoxication, indicated by necrosis of the liver and loss of coagulability of the blood, causes an increase of the proteolytic enzyme normally present in the blood serum. Increase of proteolytic activity is exhibited

in the presence of weak acid, but is much less evident in an alkaline or neutral medium. This observation suggests the possibility that the proteolytic enzyme of liver tissue, which causes autolysis and has maximum activity in the presence of acid, is freed by disintegration of hepatic cells and accumulates in the blood serum.

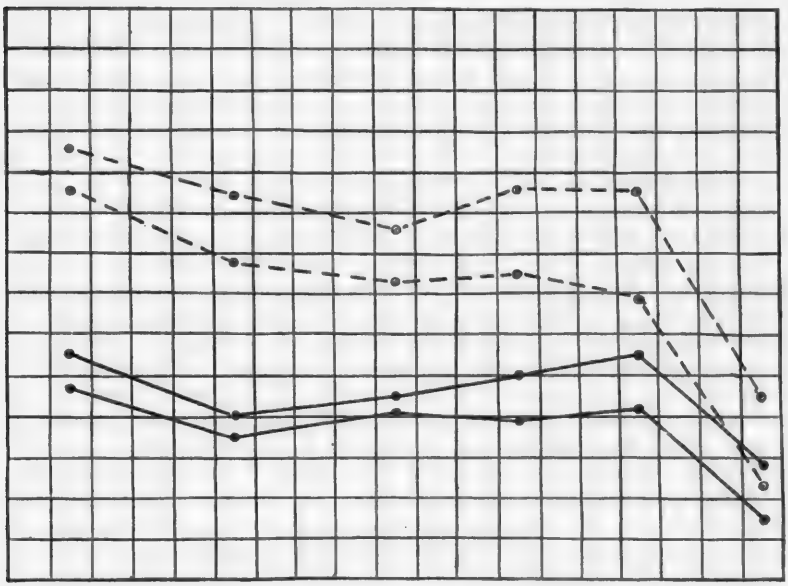

CHART 1.—Diagram showing increase of anti-enzymes in the blood serum of an animal (see Experiment 22) receiving chloroform daily. The solid lines represent digestion by enzyme of acid-treated liver in the presence of 0.25 c.c. (upper solid line) and of 0.5 c.c. (lower solid line) of blood serum. The broken lines represent digestion by leucoprotease in the presence of 0.25 c.c. (upper broken line) and of 0.5 c.c. (lower broken line) of blood serum. Changes in the two pairs of lines are an index of the activity of anti-enzyme for the two enzymes, and are, in general, parallel.

The anti-enzymotic action of the blood serum has been tested in animals which have repeatedly received chloroform in quantity insufficient to produce fatal intoxication. There is no increase of the normal power of the serum to restrain autolysis of liver. Nevertheless, when anti-enzymotic action of blood serum is tested

with leucoprotease, gradually increasing activity constantly accompanies continued administration not only of chloroform but of phosphorus as well. On the one hand, it is especially noteworthy that the enzyme of leucocytes digests protein in an alkaline or neutral medium, whereas the proteolytic enzyme of liver exhibits maximum activity in acid. On the other hand, it is now known that treatment of liver with weak acid renders active an enzyme which digests with energy in alkali in much the same way that treatment of fresh extract of pancreas with acid transforms pancreatic zymogen into trypsin. Is increase of anti-enzyme for leucoprotease an index of increase of anti-enzyme for this second enzyme of liver, which, like leucoprotease, digests protein with maximum activity in alkali, and is, perhaps, freed by disintegration of liver cells? Tests with acid-treated liver show that anti-enzyme for the hepatic enzyme which digests in alkali is increased during the progress of chloroform intoxication, and parallel tests with leucoprotease and with this hepatic enzyme have shown that serum which exhibits increased inhibition of one exhibits increased power to restrain the other.

The following scheme represents the relationship of hepatic enzymes to enzymes and anti-enzymes of the serum which is suggested by the foregoing experiments:

Proteolytic enzymes of liver.	Proteolytic enzymes of blood serum.	Anti-enzymotic action of blood serum.
(A) Enzyme digesting in acid.	(A) Enzyme digesting in acid. *Increased by chloroform.*	(A) Alkalinity of serum.
(B) Enzyme digesting in alkali. (Made active by acid-treatment of fresh liver.)	(B) Enzyme digesting in alkali. (Present in globulin fraction of serum and inhibited by anti-enzyme of albumin fraction.)	(B) Anti-enzyme for trypsin, leucoprotease, and similar enzyme of liver. *Increased by chloroform.*

Increase of anti-enzyme occurring in association with intoxication by chloroform or by phosphorus is, doubtless, similar to that which has been repeatedly observed with the cachexia of malignant growth

with various infections, and with other conditions. The increased protein disintegration which accompanies such processes is, perhaps, associated with liberation of proteolytic enzymes similar to those concerned in the postmortem autolysis of organs. The experiments which have been described suggest that formation of anti-enzyme is a means by which the body is protected from enzymes liberated by degeneration of cells.